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Influence of Microcarrier Surface Modification on Adhesion and Product Formation of Mammalian Cells

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1 Summary

Some recombinant proteins and numerous viral vaccines are currently produced by anchorage dependent cells. Proliferation of these cells occurs only after adhesion to a suitable surface. In large scale microcarriers can be used to provide such surfaces and at the same time treat the systems as suspension cultures since the carriers themselves are suspended in the bioreactor. Such processes are easier to scale up than multi unit processes as for example cultivation in roller bottles. Additionally the bioreactor allows a better control of the environmental conditions and the application of bioprocess techniques like fed batch or perfusion cultivation which greatly increase the productivity. In several publications it has been shown that product quality is improved in perfusion processes as steady state conditions are achieved and the cells do not need to adapt to constantly changing levels of nutrients and waste products as in batch and fedbatch processes. Furthermore in microcarrier cultures the attachment to a surface facilitates cell-cell contacts and a different organisation of the cytoskeleton compared to growth in suspension. It has been reported in literature and also shown in this study that the attachment to microcarriers can increase the specific productivity of cells compared to suspension culture.

Cytodex III are mainly used for the propagation of anchorage dependent cells in the vaccine industry. To facilitate cell attachment the carriers have a coating of porcine collagen. As production processes get increasingly free of animal derived raw materials it is desirable to substitute also the collagen by a chemically defined ligand of non animal origin. To this end about 100 different prototypes were tested. The prototypes were synthetised by combining five different matrices 11 spacers and ten types of ligands or surface coatings. The surface coatings included different fractions of fish gelatine as well as recombinant human gelatine. A synthetic peptide comprising the sequence RGD (arginine-glycine-aspartate) which is the core attachment motif in proteins such as collagen or fibronectin was also successfully tested in the experiments. As simple chemically defined ligands DEAE (diethyl-aminoethyl) as well as lysine and arginine and some of their derivatives were tried. Only arginine and DEAE proved to be animal free, economic viable alternatives to the porcine collagen.

Cytopore I are cellulose based macroporous carriers. Their surface charge is conveyed by DEAE and they are intended for the cultivation of anchorage dependent as well as suspension cells that are entrapped inside of the pores. The influence of the DEAE concentration on cell attachment, cell growth and recombinant protein production of CHO cells was evaluated. A minimum surface charge of about 0.5 meq/g was found necessary to allow sufficient cell carrier interaction for attachment. With increasing charge concentration the achieved maximum cell number generally decreased however the specific productivity of the cells increased and the process seemed to be more robust to deteriorating culture conditions like low pH or low nutrient concentration. Up to the maximum tested charge concentration of 1.8 meq/g no cytotoxic effect of the surface charge was detected.

CHO cells producing different recombinant antibodies were cultivated on Cytopore I and Cytoline I in a stirred tank reactor and a fluidised bed reactor respectively. The microcarrier cultivations were run as perfusion processes. For the cell line CHO 2F5 the resulting volumetric productivities and antibody concentrations were compa-

red to cultivation in batch and fedbatch culture. The composition of the culture medium was optimised to limit perfusion rate and hence increase product concentration.
The cell concentration achieved on Cytopore I and Cytoline I was three to five times higher than in fedbatch culture. The volumetric productivity of the two perfusion systems was at least six times higher. The product concentration in the microcarrier based fermentations was greatly increased by lowering the perfusion rate. On Cytoline1 it was finally equal to fedbatch while on Cytopore1 even a 30% higher antibody concentration was achieved.

During cultivation on microcarriers cells are not easily accessible for viability determination therefore often indirect methods are used to assess the state of the cells. In this study the use of lactate dehydrogenase (LDH) as marker for damage of the cell membrane was evaluated. The experiments were done for semicontinuous perfusion cultures on Cytopore I as well as for a perfusion process on Cytoline I carriers. The concentration of the enzyme in supernatant samples was stable for a storage period of 300 days at –80°C. The average amount of LDH released per CHO cell was $7.54*10^{-7}$ units. In the cultivation on Cytoline I carriers especially during the stationary phase no clear correlation between cell viability and LDH concentration was detected. However during the semicontinuous perfusion on Cytopore I carriers LDH release was a useful indicative parameter for cell viability.

Keywords: Microcarrier surface, CHO, high density perfusion culture

2 Zusammenfassung

Adhärente Zellen werden derzeit zur Produktion von einigen rekombinante Proteinen und zahlreichen viralen Vakzinen eingesetzt. Wachstum solcher Zellen findet nur statt, wenn eine geeignete Oberfläche zur Verfügung steht. Im Produktionsmaßstab werden überwiegend Microcarrier für die Kultivierung von adhärenten Zellen verwendet, da sie es erlauben den Prozess wie eine Suspensionskultur zu führen. Microcarrier basierte Kultivierung in Bioreaktoren ermöglicht einen wesentlich einfacheren Scale-up als sogenannte Multi Unit Prozesse wie zum Beispiel die Verwendung von Rollerflaschen. Darüberhinaus können die Umweltbedingungen in Bioreaktoren viel besser kontrolliert werden und die Anwendung von Techniken wie Fedbatch oder Perfusion wird ermöglicht, wodurch die Produktivität der Prozesse stark erhöht werden kann. In mehreren Publikationen wurde außerdem gezeigt, dass durch den Einsatz von Perfusionskulturen die Produktqualität verbessert werden konnte, da sich ein Gleichgewichtszustand einstellt und sich die Zellen nicht auf ständig veränderte Bedingungen wie bei Batch oder Fedbatchkultivierung einstellen müssen. Weiters fördert das Wachstum auf Microcarriern Zell-Zell Kontakte sowie einen anderen Aufbau des Cytoskeletts als in Suspensionskultur. In der vorliegenden Arbeit konnte gezeigt werden, dass durch das Wachstum auf Microcarriern die spezifische Produktivität von CHO Zellen gegenüber der Kultivierung in Suspension erhöht werden kann. Dieses Ergebnis wird auch durch bereits veröffentlichte Daten von anderen Zelllinien bestätigt.

Cytodex III Carrier werden hauptsächlich für die Anzucht von adhärenten Zellen in der Impfstoffindustrie verwendet. Sie sind mit einer Kollagenschicht überzogen, um die Zellanheftung zu erleichtern. Das Kollagen ist tierischen Ursprungs und wird aus Schlachtabfällen von Schweinen gewonnen. Da die Verwendung von tierischem Material in Produktionsprozessen immer weiter zurückgedrängt wird, sollte auch das Kollagen durch einen chemisch definierten Liganden nicht tierischen Ursprungs ersetzt werden. Um dafür geeignete Materialien zu finden wurden etwa 100 verschiedene Prototypen getestet. Diese wurden durch Kombination von fünf unterschiedlichen Matrices, 11 verschiedenen Spacern und zehn verschiedenen Liganden bzw. Beschichtungsmaterialien syntethisiert. Die getesteten Oberflächenbeschichtungen waren verschiedene Arten von Fisch Gelatine sowie humanes, rekombinantes Kollagen. Ausserdem wurde ein synthetisches Peptid, das die Sequenz RGD (Arg-Gly-Asp) beinhaltet erfolgreich getestet. Die drei Aminosäuren bilden jene Bereiche in Kollagen oder Fibronektin an die sich Zellen durch Rezeptorbindung anlagern können. Als einfache, chemisch definierte Liganden wurden DEAE (Diethyl-Aminoethyl) sowie Arginin und Lysin und einige ihrer Derivate verwendet. Dabei erwiesen sich Arginin und DEAE als preisgünstige, chemisch definierte Alternativen zu Kollagen.

Cytopore I sind makroporöse Carrier aus Zellulose. Durch eine Modifikation mit DEAE wird eine Oberflächenladung erreicht. Die Carrier können sowohl für die Kultivierung von adhärenten als auch von Suspensionszellen eingesetzt werden. Der Einfluss der DEAE Konzentration auf Zellattachment, maximal erreichte Zellzahl und spezifische Produktivität von rekombinanten CHO Zellen wurde untersucht. Es wurde festgestellt, dass eine minimale Ladung von etwa 0,5 meq/g notwending ist, um eine ausreichende Zell-Carrier Interaktion zu erreichen. Mit zunehmender Ladungskonzentration nahm die erreichte maximale Zellzahl ab allerdings kam es gleichzeitig zu einer Steigerung der spezifischen Produktivität. Daneben schien auch die Resistenz der Kulturen gegen ungünstige Bedingungen wie niedrige pH Werte oder niedrige Nährstoffkonzentrationen zuzunehmen. Bis

zu der höchsten getesteten Ladungskonzentration von 1,8 meq/g war kein zytotoxischer Effekt der Ladung sichtbar.

CHO Zelllinien, die verschiedene rekombinante Antikörper produzieren, wurden auf Cytopore I und Cytoline I Carriern in einem Rührkessel bzw. einem Fließbettreaktor kultiviert. Die Kultivierungen auf Microcarriern wurden als Perfusionsprozesse durchgeführt. Für die Zelllinie CHO 2F5 wurden die erzielten volumetrischen Produktivitäten und Antikörper Konzentrationen mit Ergebnissen aus Batch und Fedbatchkultivierungen verglichen. Die Zusammensetzung des Kultivierungsmediums wurde optimiert um die notwendige Perfusionsrate zu reduzieren und dadurch die Produktkonzentration zu steigern. Die Zellzahlen auf Cytopore I und Cytoline I Carriern waren drei bis fünfmal höher als jene in Batch und Fedbatch Kulturen. Die volumetrische Produktivität der beiden Perfusionssysteme war zumindest sechsmal höher. Die Produktkonzentration in den Kulturen auf Microcarriern konnte durch die Rücknahme der Perfusionsrate stark erhöht werden. Auf Cytoline I war sie schließlich gleich hoch wie im Fedbatch Prozess während auf Cytopore I sogar eine um 30 % höhere Antikörperkonzentration erzielt wurde.

Während der Kultivierung auf Microcarriern sind die Zellen für eine Viabilitätsbestimmung schwer zugänglich daher werden oft indirekte Methoden eingesetzt um diesen Parameter zu analysieren. In dieser Arbeit wurde die Verwendung von Lactat Dehydrogenase (LDH) als Marker für Schäden an der Zellmembran untersucht. Die Experimente wurden in semikontinuierlicher Perfusion auf Cytopore I Carriern sowie in Perfusionskultur auf Cytoline I Carriern durchgeführt. Die Aktivität des Enzyms in Proben aus dem Kulturüberstand war bei –80 °C über einen Zeitraum von 300 Tagen stabil. Die durchschnittliche LDH Menge pro CHO Zelle betrug $7{,}54*10^{-7}$ Units. In der Fermentation auf Cytoline I Carriern war vor allem in der stationären Phase kein eindeutiger Zusammenhang zwischen Viabilität und LDH Konzentration erkennbar. Bei der Kultivierung auf Cytopore I Carriern erwies sich die Konzentration des Enzyms im Überstand dagegen als nützlicher Parameter zur Abschätzung der Viabilität.

3 Introduction

Adhesion of cells to biomaterials is fundamental in many biotechnological processes. Numerous pharmaceuticals for human and veterinary use such as interferon, human tissue plasmin activator (1) and viral vaccines (influenza, hepatitis, rabies, polio, rota virus, japanese encephalitis virus, food and mouth disease) (2, 3, 4, 5) are produced by anchorage dependent cells. Proliferation of these cells occurs only after adhesion to a suitable surface. Especially in large scale cultivation the microcarrier technique has proven to be more effective than cell culture on flat substrates such as culture dishes or roller bottles. Microcarriers provide a suitable growth surface for adherent cells and allow at the same time to treat the processes as suspension cultures. This enables the use of bioprocess technologies that are not feasible when cultivating the cells on vessel surfaces. Large scale production in roller bottles is possible but requires the multiplication of vessels during scale up. This in turn can only be done using automatic roller stations. The drawbacks associated with cultivation in multiple vessels are a significantly higher risk of contamination and a relatively poor control of the process parameters in the individual vessels. As the application of microcarriers makes it possible to treat anchorage dependent cells like suspension cells scale up can be done by simply using larger vessels. 1 g of macroporous carriers has a surface area of about 1 m^2. At a carrier concentration of 2 g/l a 10 l reactor that still can be operated at laboratory scale provides the equivalent growth surface of 116 roller bottles with a surface area of 850 cm^2. Furthermore the cultivation in bioreactors allows a better control of the environmental conditions and the application of bioprocess techniques like perfusion cultivation which greatly increases the volumetric productivity. Growth on microcarriers also creates an environment that allows cells a better conditioning of their immediate surroundings due to the high cell concentration. Additionally the attachment to a surface facilitates cell-cell contacts and a different organisation of the cytoskeleton compared to the growth in suspension which can result in an increased specific productivity. In a publication of Spearman et al. (6) β-interferon expressing CHO cells that were grown on Cytopore I carriers showed a 5-fold higher specific productivity compared to suspension cultures.

3.1 Development of microcarries

The development of microcarriers started in the 1960ies when van Wezel first cultivated cells on DEAE-Sephadex beads (7). The beads were already in use as anion exchanger in chromatography. Cell attachment to the charged sephadex beads occured via electrostatic interaction. Van Wezel was thus the first one to cultivate primary cells without attachment to the vessel surface. He also recognised the potential to scale up cultures of adherent cells and performed extensive research on large scale vaccine production (8, 9). According to van Wezel a microcarrier needs to have the following properties:

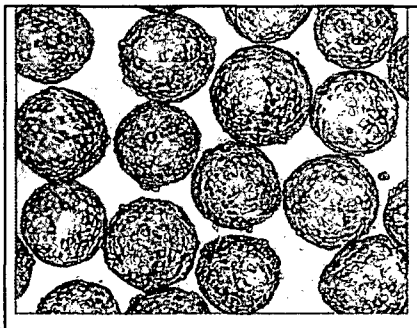
-The surface must allow cell adhesion

-The diameter should be in the range of 150 to 250 μm. A small variance of the bead size ensures that cells on all microcarriers reach confluence at the same time.

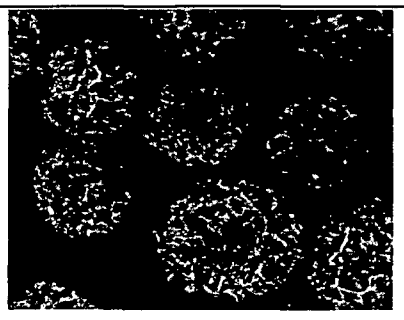
-The density of the microcarriers should be between 1.05 and 1.15 g/cm^3 to keep the carriers in suspension by relatively low stirrer speeds and thus reduce shear forces that hinder cell attachment and proliferation. However the carriers settle without stirring which allows media changes

-The carriers should be transparent to allow microscopic observations of cell growth

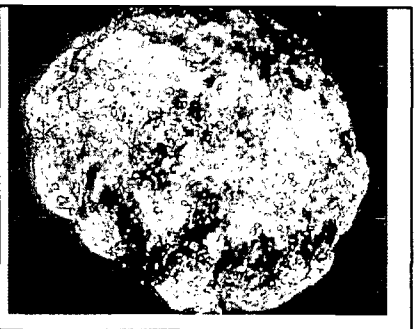
These requirements apply only to smooth or microporous carriers as cell growth takes place on the surface of the beads. Significant improvements have been made to provide microcarriers suitable for various cell types and also for different cultivation methods. Besides dextran also polystyren, polyethylen, glass and collagen were used as matrices. Carrier surfaces were also modified with extracellular matrix proteins like collagen or fibronectin. Unspecific coating by incubation with serum was practised as well. Concerns regarding product safety led to the development of coatings from non animal origin like poly lactic acid, poly L-lysine or trimethyl ammonium. A further important development was the introduction of macroporous carriers that allow cell growth inside of their pores. Besides providing a significantly larger surface area they also offer good protection of the entrapped cells against shear forces. Additionally microcarriers with higher density have been developed to allow cell cultivation not only in stirred tank reactors but also in fluidised bed reactors. In Table1 an overview of commercially available microcarriers is given.



Vero cells on Cytodex III



CHO cells on Cytopore I



CHO cells on Cytoline I

Name	Material	Surface Modification	Manufacturer
Cytodex I	dextran	DEAE	GE Healthcare
Cytodex III	dextran	gelatine coated	GE Healthcare
Cytoline I, II	polyethylen, silica		GE Healthcare
Cytopore I, II	cellulose	DEAE	GE Healthcare
Hillex	polystyrene	cationic trimethyl ammonium	SoloHill Engineering Inc.
ProNectin F	polystyrene	recombinant fibronectin	SoloHill Engineering Inc.
Biosil	polystyrene	glass coated	SoloHill Engineering Inc.
Carboseed S	carbon	cationic modification	Blue Membranes GmbH
Carboseed P	carbon	cationic modification	Blue Membranes GmbH
Biosilon	polystyrene		Nunc
Microhex	polystyrene		Nunc
Cellagen	collagen		MP Biomedicals
Cultisphere G	collagen		Percell Biolitica
Cultisphere S	collagen		Percell Biolitica
Siran	glass		Schott Glaswerke
DE 52	cellulose	DEAE	Whatman

Table1: Commercially available microcarriers

3.2 Cell attachment

Cell attachment to microcarrier surfaces is influenced by many factors. Already at the beginning of the development of microcarrers in the 1960ies it was proposed that the charge density of positively charged surfaces was the main factor for cell attachment. It has been demonstrated that serum, which was absorbed on the microcarrier surface, resulted in a lower cell attachment rate (10) although cell spreading and growth are improved (11). On microcarriers with positively charged surfaces like Cytodex I cell attachment was shown to follow first order kinetics and to rely on the electrostatic interaction between negatively charged cell surface and positively charged amino groups on the microcarriers (70). The negative charge on the cell surface is conveyed by the glycocalyx which is the carbohydrate part of glycoproteins and glycolipids embedded in the cell surface (12).
Besides DEAE and trimethylamine as charge modifier also polymers based on polyamino acids have been used as microcarrier matrix. On aminated poly(γ methyl l-glutamate) and cross linked poly(ε-lysine) it was shown that cell growth was strongly dependent upon the apparent pK_a which should be near neutral to allow cell growth (13). In this publication it has further been shown that the pK_a value rather than the amino group concentration exerts the dominant influence on cell growth.

Collagen coated carriers are almost neutral and cells attach to them via a different mechanism (14). The cells attach directly to the microcarrier surface via the binding of collagen. The binding is site limited which is to say that cells can only attach to particular sites of the microcarrier surface and other sites of the surface can only be covered by cell proliferation. As cell adhesion to collagen is facilitated by these relatively weak interactions, cells attach more slowly to collagen covered carriers than to carriers with a charged surface.

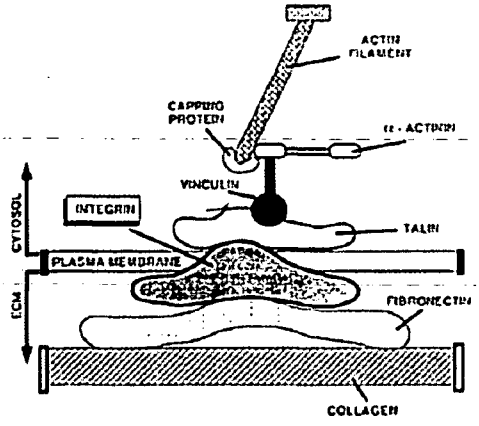


Figure1: Schematic view of cell binding to collagen (17)

Collagen coated surfaces are used frequently for establishing primary cultures and for growing cells which are normally difficult to handle like hepatocytes, fibroblasts, chondrocytes, myoblasts or epidermal cells (15, 16).
Due to its animal origin collagen is an issue of concern and there is much interest in designing synthetic substrates to replace it. Cells in tissues are surrounded by a complex network of macromolecules to which they attach via a large family of heterodimeric cell surface adhesion receptors known as integrins. Integrins are non covalently associated αβ heterodimeric cell surface receptors that recognise the extracellular matrix (ECM) or counter receptors on adjacent cells in a divalent cation dependent manner (18). In mammals 18 α and 8 β

subunits form 24 integrins (19). The bulk of the α and β subunits is extracellular, ~1000 and ~750 amino acids long respectively. Extracellular ligand recognition is determined by the specific pairing of the α and β subunit, by defined asparagine or glutamine containing sequences in ligands and by divalent cations (20). Ligand binding causes a conformational change of the integrins cytoplasmic tails and triggers intracellular events that modulate communications (mechanical or chemical) between cells and their external microenvironments (21). Cells exhibit different morphology, growth, and differentiated function depending on matrix conditions (14). The RGD (arginine-glycine-aspartic acid) sequence is known to be a key adhesion site (22) found in many proteins with which integrins interact, including fibronectin, collagen type 1, vitronectin and laminin. It has been shown that antibodies against the integrin subunits αV and β3 block the cell adhesion to vitronectin coated surfaces (23). In the same publication it was also demonstrated that RGD containing peptides blocked these receptors and inhibited cell attachment. There is intensive research going on to incorporate the RGD motif into synthetic sequences and to optimise the design of flanking regions which greatly influence cellular response after binding to RGD. The most prominent flanking sequence is the so called synergy domain in fibronectin. This is a PHSRN (Pro-His-Ser-Arg-Asn) sequence located in domain 9 of fibronectin (24). It is known to modulate RGD activity in combination with the heparin binding domain which interacts with cell surface heparan sulfate proteoglycans (14). In the same publication it was shown that the overall sequence of two synthetic RGD containing synthetic peptides greatly influenced cell spreading and cell growth on coated surfaces while the cell attachment was not influenced. The cells spread much better when a 73 kD block copolymer containing RGD units interspaced with a β silk peptide was used in comparison to a 2.3 kD RGD peptide attached to a spacer sequence. Upon contact with suitable surfaces the phenotype of the cells changes from rounded to flat and long stretched when attachment is complete. On microcarriers it is critical that this change of phenotype occurs rather quickly as cells that stay in the rounded shape adhere only loosly to the surface and are far more shear sensitive.

3.3 Cell cultivation on microcarriers

3.3.1 Inoculation of microcarrier cultures

When comparing cell attachment to the same type of surfce the attachment rate is influenced by cell concentration, microcarrier concentration and agitation speed. In literature a beneficial influence of intermitted stirring on cell attachment is reported (25, 26). However cells should ideally adhere to the microcarrier surface during continuous stirring as intermitted stirring is difficult to apply to large scale cultures. At a density of 1.04 g/cm³ Cytodex carriers settle with about 13 cm/min. With increasing vessel heigth the settling thus requires substantial time and the microcarriers at the bottom are subject to oxygen limitation. Moreover the carrier bed gets packed more tightly as bed depth increases which makes it difficult to resuspend the beads. A higher seeding density or carrier concentration supports cell adhesion because the number of cell-bead contacts during the initial phase is increased. At a given cell number for inoculation the higher carrier concentration can also be simulated by reducing the vessel volume during the inoculation phase and adding more cultivation medium after the cells have attached. The initial cell concentration is not only important in terms of the attachment rate additionally there also need to be enough cells to ensure that every microcarrier is populated. Adherent cells can cover a surface only by growth not by detachment and reattachment. Therefore carriers, which are unpopulated

at the start of the culture remain in this state during cultivation. Mendonca et al. (27) showed that for Cytodex I carriers a minimum initial ratio of 8 cells per bead is necessarry to ensure that there remain no unpopulated carriers. During the initial phase of the attachment the cells are still rounded and the relatively small contact area between cells and carrier makes them susceptible to shear stress. During the later phases of the process the cell morphology changes to flat and longstretched which allows the cells to withstand moderate shear forces. There are only few publications that actually tried to quantify the strength of cell attachment. Most of these relied on measuring the resistance of cells to liquid streams or centrifugal forces (28, 29). In a publication of Athanasiou et al. (30) a method is described which makes it possible to measure the adhesion strength of an individual cell to a surface by measuring its resistance to scraping. To this end a glass probe is positioned at the fringe of the cell using a micromanipulator. A laterally force is then applied to the glass probe and its deflection is measured by detecting the movement of an attached carbon filament using a twin fotodiode.

3.3.2 Upscaling of microcarrier cultures

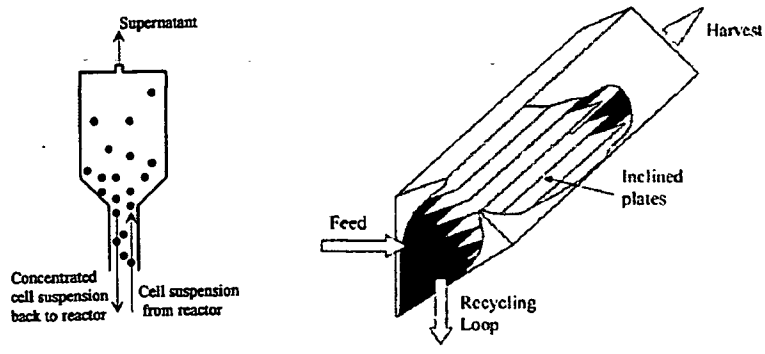
A primary task when growing adherent cells on microcarriers is the upscale of the cultures. The cells are unable to migrate between carriers therefore the addition of fresh microcarriers is usually not sufficient for upscaling the culture as the cells are unable to populate the new carriers. There is limited literature data available on upscaling a culture of Vero cells by adding fresh Cytodex III carriers (26). In this study it has been shown that Vero cells do not migrate between carriers during continuous stirring but the problem can be solved by intermitted stirring (30 minutes unstirred after every hour of agitation) for two days. As veryfied by microscopic observation the cell transfer occurred by bridging when bare beads came into contact with confluent ones. The experiments however have only been done in spinner flasks and an upscale of the procedure would be subject to the same problems that were described for the intermitted stirring during the inoculation period. To shorten the time for cell transfer and adapt the procedure to large scale requirements adherent cells are usually actively detached from the beads and afterwards have to reattach to freshly added microcarriers. The methods for cell harvesting are primarily enzymatic ones using trypsine or collagenase (26, 27). Furthermore, also the use of chelating agents like EDTA, hypotonic treatment, low temperature treatment or sonication has been described (26, 31). All of these methods damage the cells to some degree either by separating them from the extracellular matrix and thus making reattachment more difficult or by subjecting the cells to mechanical stress. The use of enzymatic methods is usually very efficient and results more or less in a single cell suspension while cell detachment by hypotonic or low temperature treatment leads to the detachment of contiguous cell sheets (31) which leaves the cells in a better condition for reattachment but makes it difficult to get an even cell distribution on the new microcarriers.

3.4 Use of microcarriers for recombinant protein production

The standard manufacturing process for recombinant proteins derived from mammalian cells still is a fedbatch process with suspension cells grown in a stirred tank reactor (32). Progress in medium development, feed strategies and cell line engineering allowed the prolongation of culture time which in turn leads to higher cell numbers and increased product concentration. Two main strategies have been employed to this end. The first is to inhibit the accumulation of toxic or inhibitory meatbolites by controlled feed strategies. For example galactose or other alternative carbon sources have been used instead of glucose to decrease lactate levels (33, 34). Besides lactate also ammonium is an undesired metabolite. It is produced from glutamine during the intracellular utilisation of the amino acid furthermore depending on the cultivation conditions some ammonium also arises from thermal decomposition of glutamine. Depending on cell line and medium, ammonium concentrations as low as 3 to 5 mM can inhibit cell growth (35, 36). A reported strategy to reduce the chemical decomposition of glutamine is the use of more stable peptides like glycyl-glutamine or GlutaMax (alanyl-glutamine, Invitrogen) (37). To reduce the ammonium generated from intracellular glutamine utilisation the amino acid has been substituted by glutamate or α-ketoglutarate (38) . In the publication of Altamirano et al. (33) a controlled galactose/glutamate feeding during a fedbatch is described which led to a duplication of culture time from 8 to 16 days compared to a glucose/glutamine feed. The second approach to increase cultivation time in fedbatch processes is the limitation of apoptotic cell death by cell line engineering like the overexpression of *Bcl-2*, $Bcl\text{-}_{XL}$ or caspase inhibitors such as *XIAP* and *CrmA* (39, 40). The benefits of cell line engineering however are obviously not limited to fedbatch cultivation but also apply to perfusion culture. Despite the extention of the cultivation time the longevity of fedbatch processes is limited due to the accumulation of toxic or inhibitory metabolites. Additionally the environmental conditions in fedbatch process deteriorate during cultivation time and can influence product formation especially folding and glycosylation. It has been shown that perfusion increases the relative glycan content and the extent of sialylation of secreted alkaline phosphatase produced in CHO cells compared to fedbatch cultivation (41). Also recombinant γ-interferon produced in perfusion cultures was found to be higher glycosylated than protein from batch cultures (42). Moreover the product is kept at cultivation temperature and is susceptible to proteases and other enzymes released by necrotic cells. For example recombinant Factor VIII is very sensitive to proteolytic degradation and high medium renewal rate of 6-8 vv/d was used in order to ensure a short residence time of the product in the reactor (43). Also for the production of β-secretase from suspension cultures of HEK 293 cells it was reported that a 75% increase in specific activity of the protein was achieved in perfusion culture compared to batch cultivation (44). The product concentration at the end of a fedbatch process is often higher than in perfusion processes however the volumetric productivity in perfusion processes and hence also the total productivity at comparable reactor size is usually about 10 times higher in perfusion processes (45, 46). As stated above in contrast to a fedbatch process the environmental conditions in a perfused bioreactor can be kept constant which often leads to better product quality and reduces the concentration of cellular enzymes and DNA in the harvest. The product is also continually removed from the bioreactor which further reduces the detrimental influence of elevated temperature and potentially released cellular enzymes.

3.5 Cell retention in bioreactors

A major challenge in the design of perfusion processes is is the reliability of the cell retention device, which should allow flexible medium renewal rates and the retention of high cell concentrations. At the same time it must be resistant to biofouling, should not pose an additional contamination risk and of course must not negatively influence cell viability and productivity. Cell retention techniques for mammalian perfusion systems include filtration via hollow fiber modules or spin filters, gravity settlers, centrifuges, hydrocyclones and acoustic separators. The retention techniques greatly vary in robustness and scalability (47, 48). Generally speaking devices relying on filtration are easy to scale up and perfusion rates of 500 to 600 l/d have been reported (49) nonetheless the systems are prone to biofouling. Centrifuges and hydrocyclones have been used for perfusion of large scale cultures and medium renewal rates of more than 3000 l/d have been published (50) however the perfusion rates are not very flexible as both devices require a minimum flow through, additionally they introduce comparably high shear forces and detrimental effects on cell viability are likely. Gravity settlers are the simplest devices to retain cells by density difference. There are two basic systems in use as shown in Figure 2. The first lets cells settle in a vertical countercurrent flow which has the prerequisite that the upward flow for medium renewal must not be faster than the cells settle. In the second type of settlers inclined parallel plates are integrated that shorten the stretch for the settling cells. The cells then slide down the inclined plates generating a countercurrent flow which enhances the settling efficiency. In general settlers are robust and do not require a minimal flow rate. On the other hand the scale up of gravity settlers is limited and so far only perfusion rates of up to 100 l/d have been reported (47). To further increase the perfusion rate a larger settler with an increased internal volume would be required. However as the process conditions, especially pH and pO_2 inside of the settler are not controlled it is desirable to keep the internal volume as small as possible. For cell cultivation on microcarriers gravity settlers are ideally suited as the carriers have a significantly higher settling velocity than the cells and therefore smaller settlers can be used. In this study the carriers were retained in a simple device relying only on the vertical countercurrent flow. The settler had an internal volume of about 150 ml and was used in a 13 l stirred tank reactor with a working volume of 5.5 to 7 liters. Perfusion rates of up to 9.5 l/d were achieved. As the device was operated inside the reactor no additional contamination risk occurred.



Vertical Settler (51) Inclined Settler (47)

Figure 2: Basic design of gravity settlers

3.6 Cell number and viability measurement in microcarrier cultures

During the cultivation on carriers especially on macroporous ones the cells are not easily accessible for cell counting and viability determination. Accurate cell numbers can easily be determined by counting the released nuclei after disintegration of the cell membrane by low pH and detergents. Classical viability testing by trypan blue exclusion requires to detach the cells which influences viability. Therefore only indirect methods can be used for viability tests. One possibility is to determine the concentration of intracellular enzymes that are released upon membrane disintegration. Various cytoplasmatic esterases, acidic phosphatase or lactate dehydrogenase (LDH) are used in this context (53, 54, 55). It was decided to make use of the latter one as NADH is used as substrate which can be measured directly. Moreover apart from cell removal no further sample preparation is necessarry. Lactate dehydrogenase is an intracellular enzyme and catalyses the transition from pyruvate to lactate as shown in Equation1. NADH is needed as electron donor.

$$NADH + pyruvate \xrightarrow{LDH} NAD^{+} + lactate$$

Equation1

Upon membrane damage LDH diffuses out of the cell and can be detected in the culture supernatant. LDH is a stable cytosolic enzyme. After cell lysis the concentration remains steady at 37 °C in culture supernatant for at least one week (56). Therefore the enzyme concentration shows a good correlation to the amount of dead cells. However it has been found that the deviation to the viability determined by hemocytometer count increases with cultivation time as the cells lyse and are therefore not counted anymore but the released LDH is still present (56). The amount of LDH released per dead cell varies with celltype and cultivation conditions. In literature the data for Vero cells range between $5*10^{-7}$ U/cell (57) and $2*10^{-6}$ U/cell (56). In the latter study it was demonstrated that cultivation conditions have a major influence on the LDH content per cell. In these experiments Vero cells were cultivated in serum free and serum containing medium. While in serum free medium the average LDH activity was $1.30*10^{-6}$ U/cell it increased to $2.11*10^{-6}$ U/cell in serum containing medium. In a study of Haslam et. al (57) L1210 (mouse lymphotic leucemia cells) cells were found to have the lowest LDH activity per cell with $1.29*10^{-6}$ U/cell. The highest LDH content in this publication was found for HF-B1 (human foreskin fibroblast cells) with $6.52*10^{-6}$ U/cell. CHO K1 cells were reported to have a LDH activity of $3.04*10^{-6}$ U/cell.
The principle of LDH assays is either to directly measure the depletion of NADH or to use NADH as electron donor in a second redox reaction where a chemical is reduced to a colored substrate for example INT (iodophenyl nitrophenyl phenyltetrazoliumchloride) to formazan. There are several commercially available kits which make use of the latter reaction like the Cytotoxicity Detection Kit or the Cytotox 96.
NADH can also be directly measured by reading the absorption at 340 nm. The NADH concentration can be determined by using the absorption at 340 nm and multiplying it with the molar absorption coefficient of the substance which is $6.23*10^{3}$ $l*mol^{-1}*cm^{-1}$. One Unit of LDH converts 1 µmol of pyruvate to lactate per minute. Therefore the LDH concentration can be assessed by measuring the speed of NADH depletion and insert it into Equation 2.

$$\frac{k[\min]}{6{,}23x10^{3}[lmol^{-1}cm^{-1}]x1[cm]}x1000xdilution = LDHconc.\left[U/ml\right]$$

Equation 2

A second possibility to directly detect NADH is by measuring the fluorescence at 465 nm after excitation at 340 nm. The LDH concentration then has to be determined according to a standard curve. Also the dynamic range of the fluorescence measurement is smaller than the measurement of the light absorption at 340 nm which makes the method less suitable if samples with large concentration differences are analysed.

4 Aims of this study

This study can be divided into two main parts. The first one consisted of the analysis of cell growth and product formation of Vero and CHO cells on various microcarriers and was done in small scale using spinner flasks and microtiterplates. The second part dealt with the process optimisation for cultivating recombinant CHO cells on macroporous carriers. For these experiments the cells were grown in bioreactors.

Development of a collagen free microcarrier with properties similar to Cytodex III

Production processes get increasingly free of animal derived raw materials. Most current processes are already operated under serum free conditions. Additionally also alternatives for porcine derived trypsine that is so far used for cell detachment are emerging. Under the given circumstances it seems likely that in the long run pressure will mount to remove also the porcine derived collagen that is used as coating on Cytodex III from production processes. We therefore tested numerous Cytodex prototypes with ligands from non animal origin for their ability to support attachment and growth of Vero cells.

Examination of the influence of carrier surface charge on cell attachment and product formation of recombinant CHO cells

Cells can attach to surfaces via electrostatic or receptor mediated interaction. In the case of Cytopore carriers DEAE is used to convey the surface charge necessary for cell attachment. Using a variety of prototypes with different charge concentration the effect of this parameter on cell attachment, cell growth and specific productivity of CHO cells was analysed. Additionally, it was tested, if any of the used surface charges exerted a cytotoxic effect.

Process development for perfusion cultivation of recombinant CHO cells based on Cytoline I and Cytopore I microcarriers

To develop a robust perfusion process using microcarriers different retention devices relying on filtration and gravity settling were tested with Cytopore I carriers. Perfusion processes are often associated with relatively low product concentrations compared to fedbatch cultivations. In order to reduce the necessary perfusion rate and thereby overcome this problem a new, more concentrated cultivation medium was developed.

Comparison of microcarrier based perfusion processes to batch and fedbatch cultivation

CHO cells were cultivated on Cytoline I and Cytopore I carriers to show that due to the increased cell concentration and hence the elevated volumetric productivity microcarrier based perfusion is a viable alternative to production in a fedbatch process.

Determination of cell viability in microcarrier cultures by analysis of lactate dehydrogenase concentration in the culture supernatant

During cultivation on microcarriers cells are not easily accessible for viability determination therefore often indirect methods are used to assess the state of the cells. In this study the use of lactate dehydrogenase as marker for damage of the cell membrane was evaluated.

5 Materials and Methods

5.1 Media

If not stated otherwise all chemicals for medium preparation were purchased from Sigma-Aldrich, Austria. Except for the CHO 2F5 cultivations the basal media for all CHO fermentations was the Polymun LS mixture which is a modified Dulbecco's Modified Eagle Medium / Ham's F12 Medium (Biochrom, Berlin, Germany), supplemented with 6 mM L-Glutamine, protein free additives (Polymun Scientific, Vienna, Austria), 0.25% soy peptone (Hy Pep 1510 Quest, Naarden, the Neatherlands) and methotrexate. For suspension cultures a concentration of 250 µM ferric citrate was used, in microcarrier cultures the concentration was lowered to 100 µM as the additive reduces cell adherence.
During cultivation cells were additionally fed with concentrates of glucose, selected amino acids and soy peptone. The exact compositions of the feed solutions are stated below.

For the two first CHO 2F5 cultivations Dulbecco's Modified Eagle Medium with Ham's F12 medium (Biochrom, Berlin, Germany) was used and supplemented with 1 g/l D-glucose (final concentration 4 g/l), 4 mM L-glutamine, protein free additives (Polymun Scientific, Vienna, Austria), 0.25% soy peptone and 1 µM methotrexate (MTX).
Due to the relatively low concentration of amino acids the DMEM/Ham's F12 was not suitable for perfusion processes. Therefore spent media analysis was done and the concentration of the amino acids shown in Table 2 was increased.

Component	Conc. [g/l]
L-Glutamine	0,584
L-Asparagine	0,139
L-Aspartate	0,157
L-Glutamate	0,157
L-Isoleucine	0,155
L-Methionine	0,067
L-Tryptophane	0,039

Table 2: amino acid supplemented for CHO 2F5 cultivation

After two CHO 2F5 cultivations the concentration of the amino acids glutamine and glutamic acid were further increased to the levels shown in Table 3. Additionally the medium was also supplemented with serine and lysine.

Component	Conc. [g/l]
L-Glutamine	0,730
L-Asparagine	0,139
L-Aspartate	0,157
L-Glutamate	0,180
L-Isoleucine	0,155
L-Methionine	0,067
L-Tryptophane	0,039
L-Serine	0,046
L-Lysine	0,154

Table 3: amino acids supplemented for CHO 2F5 cultivation

During fermentation the perfusion rate was calculated to maintain a specified level of residual glucose. To reduce perfusion rate and thus increase product concentration the medium was additionally supplemented with glucose during cultivation. In order to allow experiments with different glucose concentrations a glucose stock solution with a concentration of 10 g/l was used.

For fedbatch cultivation of CHO 2F5 the process was started using DMEM/Ham's medium supplemented with the amino acids stated in Table 3. During the feed phase the residual glucose concentration was kept at 2 g/l using the feed solution described in Table 4.

Component	final conc. [g/l]
L-Glutamat	3,71
L-Aspartat	3,36
L-Cystin	2,03
L-Asparagine	3,76
D-Glucose	27,72
Soy Peptone 25 kD, UF	3,75
DMEM/HAM's F12 (1:1)	
w/o Gln, +protein free add.	

Table 4: feed solution for fed batch cultivation

To increase the glucose concentration in CHO 2G12 cultivations the concentrate shown in Table 5 was used.

Component	final conc. [g/l]
D-Glucose	3,71
L-Asparagine*H_20	3,36
L-Cystin*HCl*H_2O	2,03
L-Glutamine	3,75
L-Prolin	27,72
Soy Peptone 25 kD, UF	3,75
HQ-water	ad 1000 g

Table 5: feed solution for CHO 2G12 cultivation

Vero cells were cultured in Dulbecco's Modified Eagle Medium with Ham's F12 medium (Biochrom, Berlin, Germany), supplemented with 4 mM L-Glutamine, 0.1% soy peptone and 0,01% β-Cyclodextrin (Roquette, France).

5.2 Microcarriers

Cytoline I carriers are lens shaped macroporous polyethylene carriers weighted by mica. They are macroporous and have a surface area of about 0.3 m^2/g, their density is 1.32 g/cm^3 which allows use at high recirculation rates in fluidised bed reactors (FBRs).
Cytopore I carriers are macroporous cellulose carriers. Due to coupled diethylaminoethyl (DEAE) groups they have a surface charge of 1.1 meq/g. The particle diameter is 200-280 µm and the surface area about 1 m^2/g carrier. Their swelling factor is 40 ml/g dry carrier and their density 1.03 g/cm^3. The relatively low density allows Cytopore carriers to be used in stirred tank reactors (STRs) as well as in spinner flasks.
Cytodex I carriers are microporous dextran carriers wich have positively charged DEAE groups distributed throughout the carrier matrix. They have an average bead diameter of 190 µm, a surface area of 0.44 m^2/g and a swelling factor of 18 ml/g dry carrier. Their density is 1.03 g/cm^3.
Cytodex III carriers are gelatine coated dextran carriers. The coating facilitates cell attachment. The carriers have a reduced swelling factor of 14 ml/g and consequently also a smaller average bead diameter of 175 µm as well as an increased density of 1.04 g/cm^3.
Both types of Cytodex carriers are suitable for cell cultivation in STRs and spinner flasks.

All microcarriers were prepared according to the manufacturers recommendations (Microcarrier cell culture principles and methods, GE Helathcare).
Briefly Cytoline I were extensively washed with reverse osmosis treated water (RO-water) to remove particles. The carriers were then depyrogenated with 0.1 M NaOH for at least 24 hours and again washed with RO-water until neutral pH was achieved. The carriers were finally autoclaved for 30 min at 121°C.

Cytopore and Cytodex carriers were washed 3 times with PBS and sterilised by autoclaving for 30 minutes at 121°C. Before inoculation the carriers were washed with cultivation medium.

5.3 Cell lines

CHO 2F5, CHO 2G12 and CHO 4E10 are genetically engineered CHO dhfr- cell lines producing anti-HIV-antibodies (IgG) (Polymun Scientific, Vienna, Austria). While CHO 2F5 and CHO 4E10 recognise epitopes on the gp 120 surface protein of HIV CHO 2G12 is directed against an epitope of gp 41.
CHO HB617 (Polymun Scientific, Vienna, Austria) is a gnetically engineered CHO dhfr- cell line producing an IgM antibody directed against GM1 gangliosid which is a ligand for natural killer cells that are part of the innate immune system.
Vero cells were derived from ATTCC (Nr. CCL 81).

5.4 Cell cultivation

5.4.1 Cultivation of CHO cells

CHO cells were derived from cryo stocks and after thawing either directly used for inoculation of spinner flasks or in the case of CHO2F5 cultivated in tissue culture flasks (Nunc, Roskilde, Denmark) and transferred to a spinner flask after 3 to 4 passages. Cells were cultivated at 37°C in an atmosphere containing 7% CO_2. During routine culture CHO cells were propagated in DMEM/Ham's in 125 ml spinner flasks (Techne) at 50 rpm. Cells were passaged twice a week. For inoculum preparation the routine cultures were scaled up to 500 ml spinner flasks (Techne) and cultivated in the medium used for fermentation.

5.4.2 Bioreactor cultivations

For fermentation cells were either cultivated in a fluidised bed reactor (Cytopilot Mini, GE Healthcare) with 300 ml Cytoline 1 carriers or in a 13 l stirred tank reactor (Chemap AG) with a working volume of 5 to 7 liters.
Both reactors were equipped with a dissolved oxygen sensor (Ingold, Germany) and a pH probe (Mettler Toledo, Switzerland). If not indicated otherwise the temperature was controlled at 37°C by an external waterbath (Haake, Germany). Peristaltic pumps were used for addition of medium, NaOH and in case of the STR also for perfusion. The perfusion rate was controlled gravimetically by the process-control unit.
For fluidised bed cultivation dissolved oxygen concentration was controlled by direct sparging of oxygen. The setpoint for the dissolved oxygen tension was 55% air saturation controlled via a PI loop without any deadband. The pH was controlled at a setpoint of 7.10 +/- 0.03 by adding 0.5 M NaOH as needed. The reactor was inoculated using cells from batch cultures prepared in spinner flasks. The cells were in exponential growth phase and

the initial cell concentration was about $2*10^6$ cells/ml microcarrier. During the first 6 hours after inoculation the carrier bed was fluidised intermittedly (1 minute 250 rpm, 30 minutes 110 rpm) to facilitate attachment of the cells. Afterwards the stirrer setpoint was 200 rpm, during cultivation it was increased up to 600 rpm to keep the carrier bed fluidised. The distributor plate supporting the carrier bed is susceptible to clogging by cells and cell debris. In order to minimise fouling the plate was backflushed by periodically reverting the sense of stirrer rotation.

For perfusion culture in the STR the concentration of Cytopore I microcarriers was 2 g/l. The reactor was inoculated with cells from batch cultures prepared in spinner flasks. The initial cell concentration in the reactor was 3 to $5*10^5$ cells/ml corresponding to $4.1*10^6$ cells/ml microcarrier. The carriers were retained in the reactor by a sieve or a gravity settler. Dissolved oxygen concentration was controlled by direct sparging of oxygen. Up to a cell concentration of 4 to $5*10^6$ cells/ml a 1 mm tube connector was sufficient at higher cell concentrations a ringsparger (14 x 1 mm) was used. The method of aeration is important as bubbles with a diameter below a critical level adhere to the microcarriers and transport them into the created foam layer, thus removing cells from the system. The setpoint for the dissolved oxygen tension was 50% air saturation controlled via a PI loop without any deadband. The pH was controlled at a setpoint of 7.10 +/- 0.03 by adding 0.5 M NaOH as needed.

5.4.3 Cultivation of Vero cells

Vero cells were derived from cryo stocks and cultivated in tissue culture flasks. During routine culture cells were passaged twice a week at split ratios from 1:3 to 1:5. To generate inoculum for experiments in spinner flasks cells were also cultivated in roller bottles (Corning Life Sciences, Schipol, the Neatherlands). Cells were cultivated at 37°C in an atmosphere containing 7% CO_2. Cell detachment in routine culture was achieved by trypsinisation (0.1% trypsin, 0.02% EDTA). Trypsine was purchased from Gibco BRL (Eggenstein, Germany), EDTA was from Sigma-Aldrich (Austria). For the inoculation of microcarriers trypsinisation was no suitable method as it weakened cell attachment and prevented cell spreading on the carriers therefore cells were detached using 0.02% EDTA dissolved in calcium and magnesium free PBS.

5.4.4 Microcarrier cultivations

Experiments with cytodex modifications were done in in 125 ml Techne flasks. To prevent sticking of the carriers to the glass surface the flasks were siliconised before autoclaving (Sigmacote, Sigma Aldrich, Austria). According to the swelling factor of the tested carriers the required amount of carrier suspension for a final concentration of 3 g/l was calculated. The carrier suspension was added to the flask together with 10 ml cultivation medium and left to equilibrate over night in an atmosphere containing 7% CO_2.

The cells were detached from the roller bottles using 20 ml 0.02% EDTA. After incubation for 10 to 15 minutes at 37°C 50 ml cell culture medium were added and the inocula from all roller bottles were pooled. A sample was drawn and cells were counted in a hemocytometer. Cell viability was determined by trypan blue exclusion. The amount of cell suspension to reach a concentration of $2*10^5$ viable cells per ml was calculated for the final volume of 40 ml. The inoculum was added dropwise to the equilibrated spinner flasks. The flasks were then stirred intermittedly with 35 rpm for 5 minutes and 0 rpm for 25 minutes for 6 hours. After that cell culture medium was

added to the final volume of 40 ml, the first sample for cell counting and microphotography was taken and the spinner flasks were put to continuous stirring at 35 rpm.
A 50 % media change was performed on the third day and every day thereafter. After settling of the carriers 20 ml supernatant were removed and 20 ml new, prewarmed medium were added. For oxygen supply and pH control aeration was done with synthetic air containing 5 % CO_2 (Linde Gas GmbH, Vienna, Austria). The air flow was 6 l/min and aeration was performed for about 15 seconds corresponding to 5 changes of volume as the headspace of the flasks is about 300 ml.
If not indicated otherwise, experiments were done in duplicates.

To speed up carrier testing a screening system was developed that is based on microcarriers immobilised in polystyrene vessels. The polystyrene was softened by acetone treatment, the carriers were added to the vessel and the solvent was washed out. The immobilised carriers were washed two times with PBS, once with cultivation medium and then incubated with medium containing Penicillin/Streptomycin in an atmosphere containing 7% CO_2. Inoculum concentration was $7*10^4$ cells/ml. The final volume was 150 µl/well. Cell growth was documented by microphotography 6h after inoculation and every day thereafter.

For the cultivation of CHO cells on Cytopore microcarriers in spinner flasks (125 ml wv, Techne) the sterile and pyrogen free flasks were filled with 30 ml medium and the necessary amount of microcarriers to reach a final concentration of 1 g/l was added. The vessels were equilibrated over night in an incubator with an atmosphere containing 7% CO_2.
CHO cells for inoculation were grown in 125 and 500 ml Techne spinner flasks. The cells were passaged twice a week at split ratios of 1:5 to 1:7
The cell concentration at inoculation was about $2*10^5$ viable cells/ml (final volume). The inoculum was added dropwise to the equilibrated spinner flasks. The flasks were then stirred intermittedly with 35 rpm for 5 minutes and 0 rpm for 25 minutes for 6 hours. After that cell culture medium was added to the final volume of 125 ml and the spinner flasks were put to continuous stirring at 45 rpm. Samples were taken 6 hours after inoculation and then every day. A 50 % media change was performed on the second day and every day thereafter. After settling of the microcarriers 60 ml supernatant were removed from the spinner flask and 60 ml fresh, prewarmed medium were added to the culture. After the media change the spinner flasks were aerated with synthetic air containing 5 % CO_2 for oxygen supply and pH adjustment. Aeration was done for about 12 seconds at a flow rate of 3 l/min. As the headspace of the flasks was estimated to be about 200 ml this corresponds to a threefold change of volume.

5.4.5 Cell cultivation on DEAE-Dextran coated microtiterplates

The dextrans were delivered as solutions in a water/ethanol mixture and were diluted using PBS. Immobilisation of the dextran was achieved by pipetting it into the microtiterplate, incubating it for 5-10 minutes, removing the supernatant and washing the plate with PBS. The plates were coated with dextran concentrations of 100 mg/ml; 10 mg/ml; 1 mg/ml; 0.1 mg/ml. Experiments were done in triplicates.

500 µl of the dextran solution were added per well to a 12 well plate (Nunc) and incubated at room temperature for 10 minutes. The dextran solution was removed and the wells washed 3 times with 1 ml PBS and an incubation time of 5 minutes at room temperature. The plates were stored covered with PBS at 4°C.
Before inoculation the plates were washed once with Vero medium containing pennicillin/streptomycin, filled with 1 ml medium and incubated for temperature and pH adjustment. Inoculum concentration was $1.25*10^5$ cells/ml corresponding to about $7*10^4$ cells per cm^2. The cells were grown in an incubator in an atmosphere containig 7% CO2 . Cell growth was followed microscopically and images were taken 24 hours after inoculation.

5.5 Analytics

5.5.1 Cell counting

During routine culture cells in spinner flasks were counted before passaging. 4ml of cell suspension were centrifuged and the cell pellet was resuspended in 1 ml 0.1 M citric acid containing 2% v/v Triton X100. After a minimum incubation period of 1.5 h the released nuclei were counted in a Multisizer 3 (Beckmann Coulter, Germany). Viability of suspension cultures was determined by the Trypan Blue exclusion method in a hemocytometer.
In Microcarrier cultures the method of cell counting was dependend on the available sample volume. In spinner cultures 1 ml of suspension was used for determination of the cell number. The carriers were left to settle, the supernatant removed and the carriers were resuspended in 1 ml of 0.1% crystal violet in 0.1 M citric acid. After a minimum incubation period of 1.5 h the released nuclei were counted in a hemocytometer.
In Fermentations more sample volume was available therefore the cell number was determined using the Multisizer 3. For Cytopore I carriers 2 times 5 ml suspension were used. The carriers were left to settle, the supernatant removed and the carriers then resuspended depending on the expected cell number in 1 to 5 ml 0.1 M citric acid containing 2% v/v Triton X100. For cell counting on Cytoline I carriers the sample was divided into two parts, the carriers were washed once with PBS and then resuspended in 1 to 5 ml 0.1 M citric acid containing 2% v/v Triton X100. After a minimum incubation period of 4 hours the released nuclei were counted in the Multisizer 3. Finally the carriers present in the sample were counted and the cell count was related to the carrier volume. 1 ml Cytoline I corresponds to 440 carriers.
During some carrier tests in 96 well plates the cell growth was measured using the general cell screening system (GCSS) (SLT Lab Systems, Austria). It consists of a multichannel plate reader and uses specially designed plates to measure cell growth by the reduction of transmitted light at 700 nm. By connecting the measurements at different time points and relating them to a standard curve cell counts and growth curves for every individual well can be obtained.

5.5.2 Product Analysis

IgG and IgM concentration were determined by sandwich ELISA. Polyclonal goat anti human IgG or IgM (Sigma-Aldrich Austria) were coated onto microtiterplates and then incubated with serial dilutions of either IgG

and IgM standards respectively or with culture supernatant samples. The IgG standards were derived by purification of the respective antibodies and subsequent protein quantification by measurement of the optical density. The IgM standard was from Sigma-Aldrich Austria. The antibodies were then detected with polyclonal HRP labelled goat anti human IgG or IgM antibodies (Zymed Laboratories, USA). For quantification of the bound labelled antibody o-phenylendiamin dihydrochlorid (Sigma-Aldrich, Austria) and hydrogen peroxide (Sigma-Aldrich, Austria) were added. The optical absorbance at 492 nm and at the reference wavelength of 620 nm was then measured in a multiwell plate reader (SLT Rainbow, SLT Labinstruments, Austria.).

5.5.3 Analysis of Glucose, Lactate, Glutamine and Glutamate

These parameters were routinely analysed enzymatically using a YSI 7200 (Yellow Spring Instruments, Ohio, USA).

5.5.4 Amino Acid Analysis

Amino Acid concentrations in supernatants except for those of cytsteine and proline were determined by HPLC (HP 1090 Series II Liquid Chromatograph: Hewlett Packard, Austria). Samples were filtered (0.22 µm Millex G, Millipore) and mixed with an internal standard (β-thienylalanin, Sigma). After addition of 350 µl sulfosalicylic acid (5 %) for protein precipitation, samples were filtered again and diluted with HQ water if necessary. Injection of 30 µl was done directly from microvials via an autosampler. The guard column was LiChrosper 100 RP-18 (4x4 mm, 5 µm) the column was Hypersil ODS (100x4 mm, 3 µm Hewlett Packard). The solvent was 50 mM sodium acetate (pH 5.5), acetonitrile was used for elution. The amino acids were detected by a flourescence HPLC monitor (Shimadzu RF-535) at an emission wavelength of 450 nm.

5.5.5 Analysis of the lactate dehydrognease concentration in the culture supernatant

The concentration of lactate dehydrogenase can be determined by following the rate of NADH depletion. All measurements were done in a 100 mM Tris/HCl buffer at pH 7.5. The final NADH concentration was 0.17 mM the final pyruvate concentration was 0.2 mM. Tris, NADH and sodium pyruvate were purchased from Sigma-Aldrich Austria. The assay volume was 1 ml the sample volume was varied between 50 and 500 µl, NADH and pyruvate were prepared as 20 x stock solutions and 100 mM Tris/HCl buffer was used to bring the volume to 1 ml. Analysis was done in a photometer (Hewelett Packard HP 8453). The measuring chamber was temperated at 37°C and the cuvette was stirred during the measurement. The absorption at 340 nm was measured for 3 minutes with a reading every 20 seconds. The absorption values were then plotted versus time. The slope of the graph was used to calculate the LDH concentration according to Equation 3. The molar absorption coefficient of NADH is 6,23×10E+03 l/(mol×cm).

$$\frac{k[\min]}{6{,}23 \times 10^{3}[l mol^{-1} cm^{-1}] \times 1[cm]} \times 1000 \times dilution = LDHconc.\left[\frac{U}{ml}\right]$$

Equation 3

A second possibility to directly detect NADH is by measuring the fluorescence at 465 nm after excitation at 340 nm. The dynamic range of the fluorometer was smaller than the one of the photometer therefore the NADH concentration had to be increased to 0.85 mM. The pyruvate concentration was kept at 2 mM and the Tris/HCl buffer concentration at 100 mM. The assay volume was 250 µl consisting of 150 µl sample diluted at least 1:2 in Tris/HCl buffer and 50 µl of NADH and pyruvate stock solution. Analysis was done in 96 well black plates (Nunc Roskilde, Denmark) in a fluorometer (Spectra Max Gemini XS, Molecular Devices, Germany). The measuring chamber was warmed to 37°C. The depletion of NADH was followed over 10 minutes with a reading every 20 seconds. The well contents was mixed between the readings by 3 seconds shaking.
In contrast to the absorption measurement the LDH concentration can not be calculated directly from the fluorescence but needs to be compared to a standard. LDH prepared from rabbit muscle (Sigma-Aldrich Austria) was used. The preparation was sold preweighed with certified enzymatic activity.

5.6 Microscopy

Cell morphology on Cytodex microcarriers was documented by microphotographs taken at 40 or 100 fold magnification using an Olympus IMT-2 microscope and an Olympus DP-11 digital camera.
Colonisation of Cytoline carriers was visualised by confocal microscopy. After sampling the carriers were washed with PBS and stained with 0.01% Acridinorange solution (Sigma-Aldrich, Austria). The microcarriers were then examined with a laser confocal microscope (Biorad, Austria) at a wavelength 530 nm after excitation at 488 nm.

5.7 Mathematics

Growth rate

The specific growth rate µ was determined as the increase in the ln of the cell number per day. For perfusion systems it was assumed that all cells are retained in the reactor.

$$\mu = \frac{\ln x_2 - \ln x_1}{t_2 - t_1}$$

where x_2 and x_1 are the cell numbers at times t_2 and t_1 and $t_2 - t_1$ is the time interval between sampling.

Doubling Time

$$t_D = \frac{\ln 2}{\mu}$$

Dilution Rate / Perfusion ratio

Dilution rates [d^{-1}] were defined as the quotient between the flow rate F [ml/d] and the working volume V_R [ml]. of the bioreactor

$$D = \frac{F}{V_R}$$

As the fluidised bed reactor is an inhomogeneous system and the cells are retained in the microcarrier compartment the dilution rate is related to the volume of settled microcarriers V_C [ml].

$$D = \frac{F}{V_C}$$

Consumption / Production Rates

The consumption and production rates were calculated according to the general mass balance equation:

$mass_{in} - mass_{out} + mass_{generated} - mass_{consumed} = mass_{accumulated}$

$$Q_S = \left(S_0 - \frac{(S_1 + S_2)}{2} \right) \times D - \left(\frac{S_2 - S_1}{t_2 - t_1} \right)$$

$$Q_P = \left(\frac{P_1 + P_2}{2} \right) \times D - \left(\frac{P_2 - P_1}{t_2 - t_1} \right)$$

where Q_S and Q_P are the volumetric consumption and production rates [mg/(l x d)], S_0 is the initial substrate concentration, S_1, S_2 and P_1, P_2 are substrate and product concentrations at times t_1 and t_2 respectively.

Viable Cumulative Cell Days

The viable cumulative cell days (VCCD) is the integral of the viable cell count over process time [c x d]

$$\bar{V}CCD = \int X_V \bullet dt$$

$$VCCD = \sum_{i=1}^{n} \frac{(X_{i+1} - X_i) \times (t_{i+1} - t_i)}{\ln(X_{i+1}) - \ln(X_i)}$$

$$VCCD = \sum_{i=1}^{n} \frac{X_{i+1} - X_i}{\mu_{(X_i \to X_{i+1})}}$$

Specific Productivity

The specific productivity (q_P) is the amount of product formed per cell in one day.

$$q_P = \frac{\Delta P}{\int X \bullet dt}$$

Volumetric Productivity

The volumetric productivity (Q_P) is the amount of product formed per day in relation to the reactor working volume [$mg*l^{-1}*d^{-1}$]

$$Q_P = \frac{1}{V} \times \frac{\Delta P}{\Delta t}$$

Space time yield

The space time yield is the volumetric productivity between a given timepoint during cultivation and the start of the culture [$mg*l^{-1}*d^{-1}$]

6 Results

6.1 Experiments with Cytodex Microcarriers

The objective of these tests was to characterise cell attachment and growth on modifications of Cytodex Carriers. Vero cells were grown on numerous microcarrier prototypes in order to find a spacer – ligand combination that gives a performance equal to gelatine coating.

The tested prototypes varied in matrix composition as well as in the type of spacer and ligand as shown in Table 6. By using different matrices and spacers it was tried to couple a higher ligand concentration to the carriers.

Matrix	Spacer	Ligand
Sephadex G50	AGE	Arg
Sephadex G25	PVA	DEAE
	ECH	
	BPR-Butane	
	Dextran 40k	
	Dextran 500k	

Table 6: Types of matrices, spacers and ligands used for prototype testing in spinner flasks

Additionally to the ligands mentioned in Table 6 also prototypes coated with different types of gelatine were used. As subsitute for the pig gelatine the Sephadex G50 matrix was coated with different types of fish gelatine, human recombinant gelatine and a synthetically produced RGDS (arginine, glycine, aspartic acid and serine) which comprises the core motif in the cell attachment sequence on gelatine.

Matrix: Sephadex is a bead formed gel prepared by crosslinking dextrane with epichlorhydrin. Different types of sephadex vary in their degree of crosslinking and hence in their degree of swelling. The G25 material has a lower swelling volume and therefore a higher ligand concentration per ml gel can be achieved. The reduced swelling however also results in a higher density consequently a higher stirring speed is required to keep the microcarriers in suspension.

Spacer: Spacers are necessary to allow the coupling of ligands to the base matrix. The following spacers were used:

AGE (Allyl Glycidyl Ether)

Figure 3: chemical structure of AGE

BPR-Butane

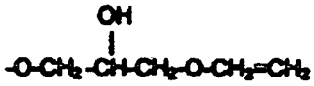


Figure 4: chemical structure of BPR-Butane

Epichlorhydrine

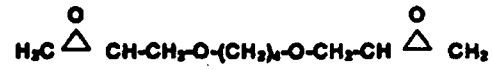


Figure 5: chemical structure of Epichlorhydrine

PVA (Polyvinylalcohol)

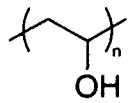


Figure 6: chemical structure of polyvinylalcohol

Dextran

Dextran is an α-D-1,6-glucose-linked glucan with side-chains 1-3 linked to the backbone units of the Dextran bio-polymer.

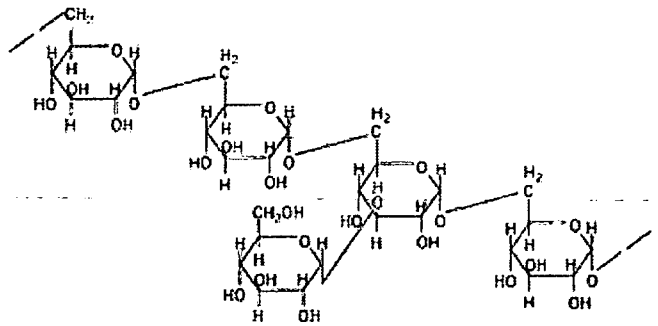


Figure 7: chemical structure of dextran

Ligands finally allow the interaction of cells with the microcarriers. They can either act by electrostatic interaction like the DEAE or cells can attach to the ligands by receptor mediated binding which should be the case for arginine as it is the first amino acid of the RGD (arginine – glycine – aspartic acid) motif to which cells attach via integrins.

DEAE (Diethylaminoethyl)

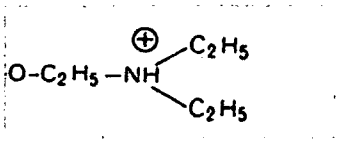


Figure 8: chemical structure of DEAE

Arginine

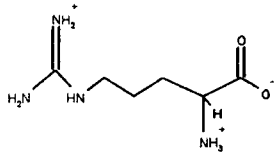


Figure 9: chemical structure of arginine

Cytodex I and III as reference carriers provide both of the interaction possibilities mentioned above. Cytodex I consists of crosslinked dextran. Positive charges are distributed througout the matrix by coupling of DEAE.

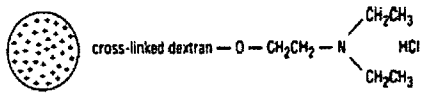


Figure 10: schematic view of Cytodex I

Cytodex III consists of the same base material and is covered with a layer of denatured collagen. Cells are known to interact with the RGD motif that is present in collagen type 1 (14).

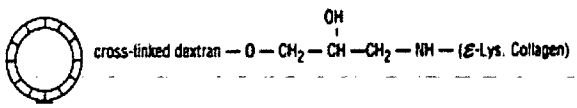


Figure 11: schematic view of Cytodex III

The negative control to ensure that cells do not attach to the spacer alone was a carrier coated with AGE only few cells did attach to this prototype and no cell growth was observed.
In Table 7 an overview of the Cytodex prototypes tested in spinner flasks is given.

Carrier	Chapter.	CC 72h	Norm to Cyt. III	max CC	Norm to Cyt. III	Spacer [µM/ml gel]	Ligand [µM/ml gel]	Annotations
Arg/Allyl								
U957011	6.1.1	3,80E+05	0,76	8,43E+05	0,86	202	43	Arg, AGE, G50
U957012	6.1.1	5,17E+05	1,03	1,38E+06	1,40	129	36	Arg, AGE, G50
U957033	6.1.1	3,00E+05	0,69	2,09E+06	0,86	113	35	Arg, G50
U957035	6.1.1	3,57E+05	0,82	2,12E+06	0,87	113	45	Arg, G50
U957062	6.1.4	1,34E+05	0,25	n.d.	n.d.	125	76	Arg, G25, Spacer - Ligand conc estimated from plot
U957073	6.1.4	7,29E+04	0,14	n.d.	n.d.	147	74	Arg, G25, Spacer - Ligand conc estimated from plot
U957076	6.1.4	5,04E+04	0,09	n.d.	n.d.	147	43	Arg, G25, Spacer - Ligand conc estimated from plot
U957077	6.1.4	2,35E+05	0,43	n.d.	n.d.	147	100	Arg, G25, Spacer - Ligand conc estimated from plot
U957080	6.1.4	4,44E+04	0,08	n.d.	n.d.	210	63	Arg, G25, Spacer - Ligand conc estimated from plot
U1309051	6.1.3	7,18E+05	0,81	2,92E+06	1,13	129	12	Arg, G50
U1390075	6.1.5	4,66E+05	0,47	n.d.	n.d.	185	71	Arg, G50
U1309076	6.1.5	n.d.	n.d.	n.d.	n.d.	185	20	Arg, G50
U1309077	6.1.5	n.d.	n.d.	n.d.	n.d.	185	53	Arg, G50
U1309078	6.1.5	n.d.	n.d.	n.d.	n.d.	185	44	Arg, G50
U1309079	6.1.5	n.d.	n.d.	n.d.	n.d.	146	42	Arg, G50
U1309080	6.1.5	n.d.	n.d.	n.d.	n.d.	125	40	Arg, G50
U1309082	6.1.5	8,71E+05	0,96	2,24E+06	1,07	146	57	Arg, G50, reached max. Cellcount 1 day later than Cyt. III
U1309085	6.1.5	n.d.	n.d.	n.d.	n.d.	146	24	Arg, G50

Carrier	Chapter.	CC 72h	Norm to Cyt. III	max CC	Norm to Cyt. III	Spacer [µM/ml gel]	Ligand [µM/ml gel]	Annotations
U1309086	6.1.5	n.d.	n.d.	n.d.	n.d.	125	29	Arg, G50
U1309087	6.1.5	4,45E+05	0,49	1,71E+06	0,82	125	44	Arg, G50, reached max. Cellcount 1 days later than Cyt. III
U1309089	6.1.5	n.d.	n.d.	n.d.	n.d.	185	39	Arg, G50
U1309092	6.1.5	5,85E+05	0,64	2,01E+06	0,96	125	46	Arg, G50, reached max. Cellcount 2 days later than Cyt. III
Arg(Epoxy)/Epichlorhydrin								
U784094	6.1.1	1,29E+05	0,26	8,10E+05	0,82	72	37	Arg(Epoxy),G50, Epichlorhydrin
Arg(Epoxy)/BPR Butane								
U957026	6.1.1	1,95E+05	0,45	1,17E+06	0,48	120	66	Arg(Epoxy), G50, BPR butane
U957034	6.1.1	4,63E+05	1,04	1,92E+06	0,79	133	83	
U957037	6.1.1	1,33E+05	0,31	n.d.	n.d.	78	51	
U957038	6.1.1	1,17E+05	0,27	n.d.	n.d.	78	62	
DEAE/Dextran 40k								
U1453040	6.1.6	1,16E+05	0,21	n.d.	n.d.	35,7	43	DEAE, G50, Dextran 40k as Spacer
U1453045	6.1.6	8,32E+04	0,15	n.d.	n.d.	36,7	51	DEAE, G50, Dextran 40k as Spacer
U1453048	6.1.6	4,19E+05	0,77	1,08E+06	1,07	44	75	DEAE, G50, Dextran 40k as Spacer
DEAE/Dextran 500k								
U1453054	6.1.6	1,32E+04	0,02	n.d.	n.d.	n.d.	68	DEAE, G50, Dextran 500k as Spacer

Carrier	Chapter.	CC 72h	Norm to Cyt. III	max CC	Norm to Cyt. III	Spacer [µM/ml gel]	Ligand [µM/ml gel]	Annotations
DEAE								
U1453052	6.1.6	2,82E+04	0,05	n.d.	n.d.	n.d.	45	DEAE first coupled to spacer then Spacer (Dextran 500k) coupled to G50
DEAE/PVA								
U1453050	6.1.6	1,10E+05	0,20	n.d.	n.d.	n.d.	26	DEAE, G50, PVA as Spacer
U1453051	6.1.6	5,96E+05	1,10	1,13E+06	1,11	n.d.	40	DEAE, G50, PVA as Spacer
Gelatine (fish)								
U784097	6.1.1	5,89E+05	1,17	1,32E+06	1,34			cold water fish
U957007	6.1.1	6,93E+05	1,38	1,73E+06	1,75			warm water solube
U957031	6.1.1	4,70E+05	1,08	2,44E+06	1,00			warm water solube
rec. Collagen								
U957042	6.1.2	5,54E+05	1,07	1,26E+06	1,06			
0,006 gr HU4/g G50	6.1.2	8,85E+04	0,12	n.d.	n.d.			neg. result; coating problems
0,009 gr HU4/g G50	6.1.2	1,32E+05	0,18	n.d.	n.d.			neg. result; coating problems
0,044 gr HU4/g G50	6.1.2	7,80E+04	0,10	n.d.	n.d.			neg. result; coating problems
0409 MC 45A	6.1.2	5,38E+05	0,71	1,40E+06	0,77			CC after 82 h
0409 MC 45B	6.1.2	5,59E+05	0,74	1,65E+06	0,91			CC after 82 h
0409 MC 45D	6.1.2	5,85E+05	0,77	1,29E+06	0,71			CC after 82 h
0410 MC 51A	6.1.2	6,74E+05	0,89	1,36E+06	0,75			CC after 82 h

Carrier	Chapter.	CC 72h	Norm to Cyt. III	max CC	Norm to Cyt. III	Spacer [µM/ml gel]	Ligand [µM/ml gel]	Annotations
0410MC 51C	6.1.2	6,23E+05	0,82	1,28E+06	0,70			CC after 82 h
RGDS								
U1309050	6.1.3	8,57E+05	0,92	4,20E+06	1,70	101	12	very strong aggregate formation

Table 7: Overview of all Cytodex prototypes tested in spinner flasks. The cellcount after 72h and if appropriate also the maximum cell count were normalised to the cell concentrations on Cytodex III in the respective experiment.

Carrier	Chapter	Cell Growth	Spacer	Spacer [µmo/ml Gel]	Ligand	Ligand [µmo/ml Gel]	Matrix	Annotations
DEAE								
U1453040	6.1.6	-	DX 40	35,7	DEAE	43	Sephadex G50	GCSS
U1453045	6.1.6	-	DX 40	36,7	DEAE	51	Sephadex G50	
U1453048	6.1.6	+	DX 40	44	DEAE	75	Sephadex G50	
U1453050	6.1.6	-	PVA	n.d.	DEAE	26	Sephadex G50	
U1453051	6.1.6	+	PVA	n. d.	DEAE	40	Sephadex G50	
U1453052	6.1.6	-	DEAE-DX	n. d.	DEAE	45	Sephadex G50	
U1453054	6.1.6	-	DEAE-DX	n. d.	DEAE	68	Sephadex G50	
U1574035	6.1.6	-	PVA	n. d.	DEAE	26	Sephadex G50	
U1574044	6.1.6	+/-	PVA	9	DEAE	45	Sephadex G50	
U1574045	6.1.6	+	DX 40	42	DEAE	63	Sephadex G50	
U1574054	6.1.6	-	none	0	DEAE	27,5	Sephadex G50	
U1574055	6.1.6	-	none	0	DEAE	50	Sephadex G50	
U1574056	6.1.6	-	none	0	DEAE	24	Sephadex G50	
U1574062	6.1.6	+	DX 40	11,2	DEAE	115	Sepharose 4FF	
U1574065	6.1.6	+	none	0	DEAE	39,5	Sepharose 4FF	
U1574066	6.1.6	-	DX 40	42	DEAE	110	Sephadex G50	
U1689080J	6.1.6	-	Allyl	259	Dx-DEAE	205	Seph 6FF	

Carrier	Chapter	Cell Growth	Spacer	Spacer [µmo/ml Gel]	Ligand	Ligand [µmo/ml Gel]	Matrix	Annotations
Arg								
U1574041	6.1.6	-	DX 40	142	Arg	40	Sephadex G50	
U1574042	6.1.6	-	DX 40	142	Arg	42,5	Sephadex G50	
U1453096	6.1.6	-	DX 40	182	Arg	49	Sephadex G50	
U957062	6.1.6	-	Allyl	125	Arg	76	Sephadex G25	Feb 04 neg. tested in spinner
U957077	6.1.6	-	Allyl	147	Arg	100	Sephadex G25	Feb 04 neg. tested in spinner
U1574016	6.1.6	-	DX 40	100	Arg	27	Sephadex G50	
U784091	6.1.6	-	Allyl	191	arg	31	Sephadex G50	GCSS
U957002	6.1.6	-	Allyl	191	arg	27	Sephadex G50	
U1574063	6.1.6	-	Allyl	318	Arg	70	Sephadex G50	
U1661003/1	6.1.6	-	Epoxy	63	arg	30	Sephadex G50	
U1689080A	6.1.6	-	Epoxy	n. d.	Arg	58	Seph 6FF	
U1689080B	6.1.6	-	Epoxy	n. d.	Arg	103	Seph 6FF	
U1689080C	6.1.6	-	Epoxy-Allyl Dextran 150 k	80	Arg	33	Seph 6FF	
U1689080D	6.1.6	-	Epoxy-Allyl Dextran 150 k	80	Arg	62	Seph 6FF	
U1689080E	6.1.6	-	Epoxy	n. d.	Arg	31	Seph Q 6FF	182 µm/ml Seph Q FF
U1689080F	6.1.6	-	Epoxy-Allyl Dextran 150 k	n. d.	Arg	40	Seph Q 6FF	182 µm/ml Seph Q FF
U1689080G	6.1.6	-	Epoxy-Allyl Dextran 150 k	n. d.	Arg	48	Seph Q 6FF	182 µm/ml Seph Q FF
U1689080H	6.1.6	-	Dx + Allyl	256	Arg	91	Seph 6FF Dextran	
U169221A	6.1.6	-	Mercapto pro-pionic acid	250	Arg	45	Seph 6FF	coupled with N-hydroxy succinimide
U169221B	6.1.6	-	COOH, 250	250	Arg	90	Seph 6FF	coupled with N-hydroxy succinimide
U169222	6.1.6	-	COOH, 250	250	Arg	15	Seph 6FF	activated for amide coupling by DMT-MM
U169224A	6.1.6	-	COOH, 250	250	Arg	100	Seph 6FF	activated for amide coupling by HATU
U169224B	6.1.6	-	COOH, 250	250	Arg	40	Seph 6FF	activated for amide coupling by TOTT

Carrier	Chapter	Cell Growth	Spacer	Spacer [µmo/ml Gel]	Ligand	Ligand [µmo/ml Gel]	Matrix	Annotations
Other								
U1403040A	6.1.6	-	Allyl	102	Lysine	21	Sephadex G50	GCSS
U1403048A	6.1.6	+/-	Allyl	102	H-Arg-Ome	15	Sephadex G50	
U169231A	6.1.6	-	COOH	250	H-Arg-OEt	55	Seph 6FF	activated for amide coupling by DMT-MM
U1403048B	6.1.6	-	Allyl	102	Agmatine	40	Sephadex G50	
U169228	6.1.6	-	COOH	250	Agmatine	190	Seph 6FF	coupled with N-hydroxy succinimide
U169229B	6.1.6	-	NH2	147	Fmoc-Arg-OH	127	Seph 6FF	
U169230B	6.1.6	-	NH2	147	Fmoc-Arg-OH	n. d.	Seph 6FF	
U169231B	6.1.6	-	NH2	147	Fmoc-Arg-OH	90	Seph 6FF	activated for amide coupling by DMT-MM

Table 8: Overview of all Cytodex prototypes tested in microtiterplates

As shown in Table 7 in total 46 Cytodex prototypes were tested in spinner flasks.

27 of these had arginine as ligand. The ligand concentration ranged from 12 to 100 µmol/ml gel. 22 of these prototypes were produced with AGE as spacer. Of these 22 Cytodex modifications 5 had Sephadex G25 as base matrix for the rest Sephadex G50 was used. The experiments with Sephadex G25 were done because the material has a lower swelling factor and therefore allows the coupling of a higher ligand concentration. On four carrier modifications BPR-Butan was used as spacer and arginine as ligand. The ligand concentrations ranged from 50 to 80 µmol/ml gel. Finally one prototype with epichlorhydrin as spacer and arginine as ligand with a concentration of 37 µmol/ml gel was tested.

On 7 Cytodex modifications DEAE was used as ligand. Three of these had dextran 40k as spacer, the DEAE concentrations were in the range of 40 to 75 µmol/ml gel. Two prototypes had PVA as spacer with a DEAE concentration of 26 and 40 µmol/ml gel. For the last two tested carriers a dextran 500k spacer was used. The DEAE concentration was 45 and 68 µmol/ml Gel.

Furthermore 3 carriers coated with fish gelatine, 9 coated with human recombinant gelatine and 1 with the aforementioned RGDS peptide as ligand were also tested in spinner flasks.

6.1.1 Experiments with fish gelatine coatings on Cytodex prototypes

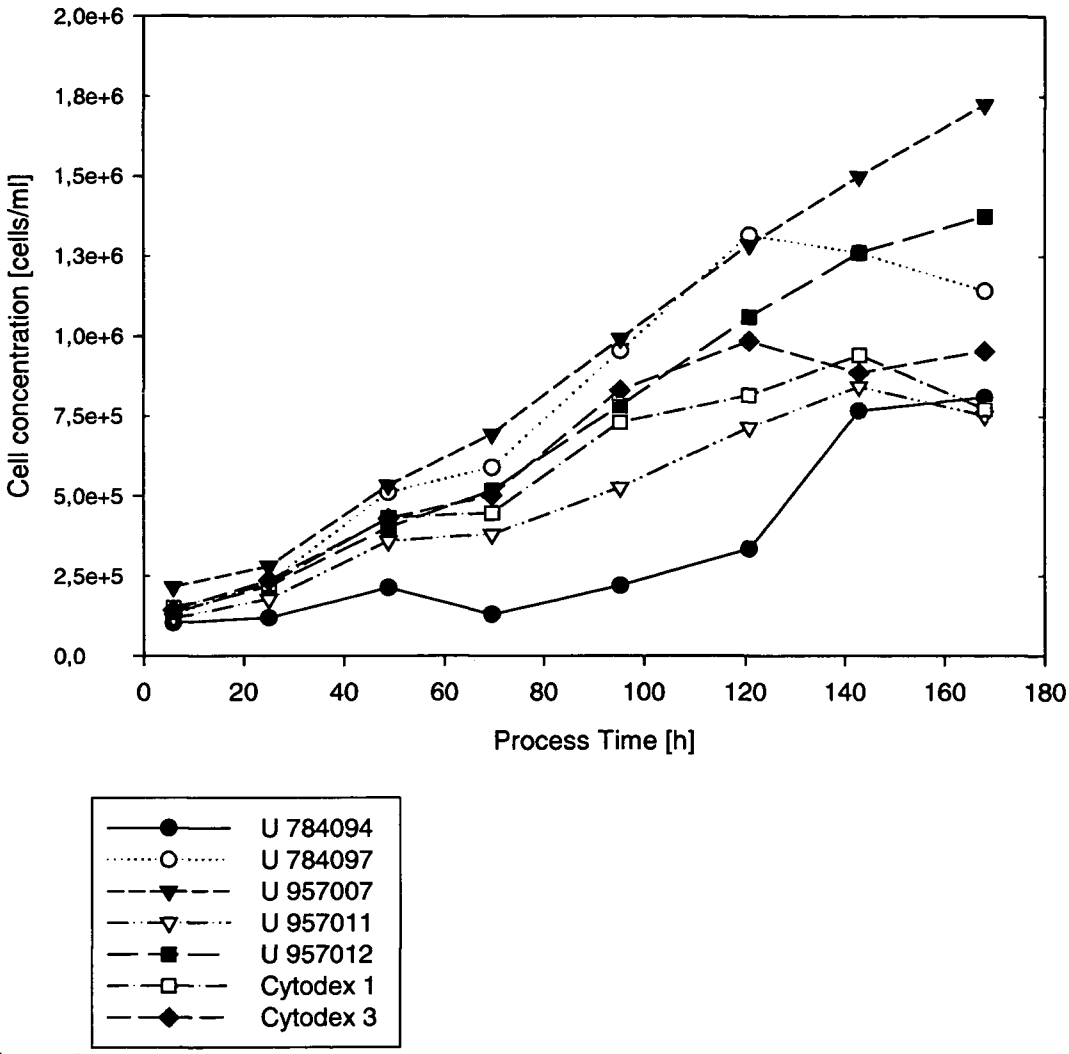


Figure 12: Growth curves Cytodex prototypes

The two carriers with the best performance in this experiment were U957007 and U784097 as shown in Figure 12. Both prototypes consist of a sephadex G50 matrix with a fish gelatine coating. The cell counts were practically equal up to a process time of 120 h. After that cell growth on U957007 was significantly better. The difference between the two Cytodex modifications is that U784097 was coated with gelatine derived from cold water fish wheras U957007 had a coating with a warm water soluble fish gelatine fraction.

For U957012 arginine was used as ligand and an allyl group as spacer. The arginine concentration was 36 µmol/ml gel. As shown in Table 7 it was the only arginine carrier that had a higher cell number than the reference carrier Cytodex III after 72 h cultivation. The maximum cell number was even 140% of Cytodex III. On the remaining two prototypes U784094 and U957011 the cell numbers did not reach the level of the reference carriers. This is especially distinct for U784094 which was the only tested Cytodex modification where epichlorhydrin was used as spacer. As can be seen in Figure 12 there was almost no cellgrowth on this carrier up to 120 h of process time. The sudden increase at 140 h was most probably caused by the deposition of extracellular matrix and hence conditioning of the carrier surface which then allowed the cells to grow. U957011 was an argi-

nine carrier with an allyl group as spacer. The arginine concentration was 43 µmol/ml gel which is similar to the the 36 µmol/ml gel on U957012. A significant difference between the two prototypes was however the spacer concentration. While on U957012 an allyl concentration of 129 µmol/ml gel was sufficient to bind 36 µmol/ml gel of arginine on U957011 202 µmol/ml of allyl were necessary to bind 43 µmol/ml arginine. Consequently there was a higher amount of unoccupied spacer on U957011. Although free reactive groups on the spacers are normally blocked during the production process an influence on cell attachment or –growth cannot be ruled out.

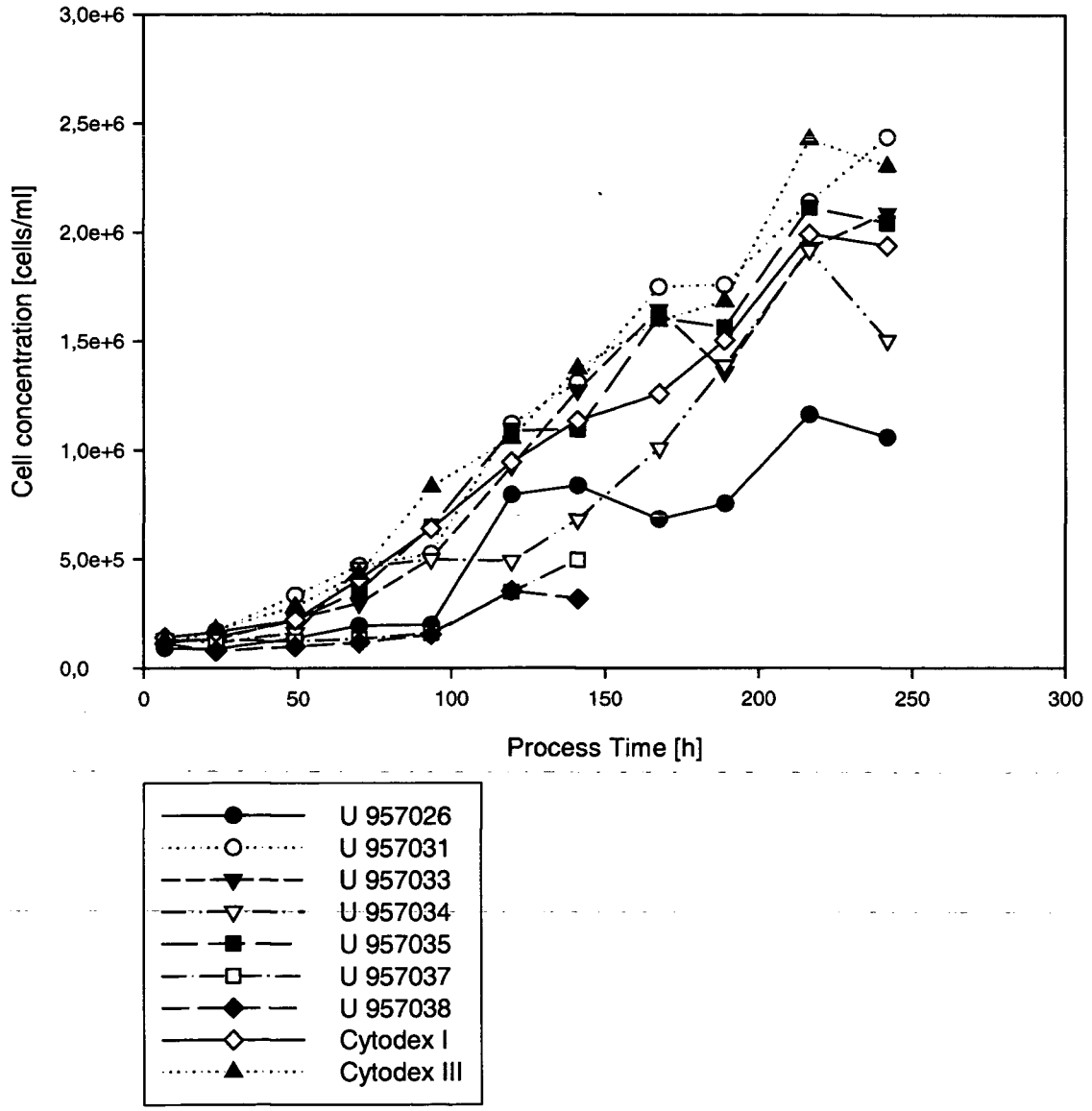


Figure 13 : Growth curves Cytodex prototypes

Cell growth on U957031 was comparable to Cytodex III. The cell count was 108% of Cytodex III after 72h and the maximum cell count was exactly equal to Cytodex III although it was reached one day later than on the reference carrier (see Figure 13). U957031 consisted of a sephadex G50 matrix covered with a warm water soluble fish gelatine fraction. Compared to U957007 which was also covered with warm water soluble gelatine and reached a maximum cell count of 175% of Cytodex III the performance of U957031 was lower. The two proto-

types U957033 and U957035 reached a cell count between Cytodex III and Cytodex I. Both prototypes used arginine as ligand and allyl as spacer. The spacer concentration was 113 µmol/ml gel in both cases, the ligand concentration of U957033 was 35 µm/ml gel. U 957035 had a higher ligand concentration of 45 µmol/ml gel.
The prototypes U957034 and U957026 had a significantly longer lag phase than the reference carriers. U957034 reached the same cell count as Cytodex I after 10 days but was below the reference carriers between 96 and 240 h. The increase in cell number presumably occured due to deposition of extracellular matrix and the resulting conditioning of the carrier surface. U957026 reached only half the maximum cell number of Cytodex III. On U957037 and U957038 almost no cell growth ocurred and the experiments were terminated after 6 days. All 4 prototypes had BPR-Butane as spacer the arginine concentrations varied from 51 to 83 µmol/ml gel.

6.1.2 Experiments with recombinant human gelatine

The fish gelatine coated prototypes showed very promising results but did not overcome the problems related to coating materials of animal origin. Therefore a novel coating strategy using human recombinant collagen was tried. As the recombinant protein is far more expensive than the standard material several prototypes were manufactured to optimise the coating procedure and find the minimal necessary amount of recombinant collagen.

U957042 was tested with Cytodex III as reference carrier. This prototype was intended as preliminary experiment and no effort was made to reduce the coated amount of collagen.

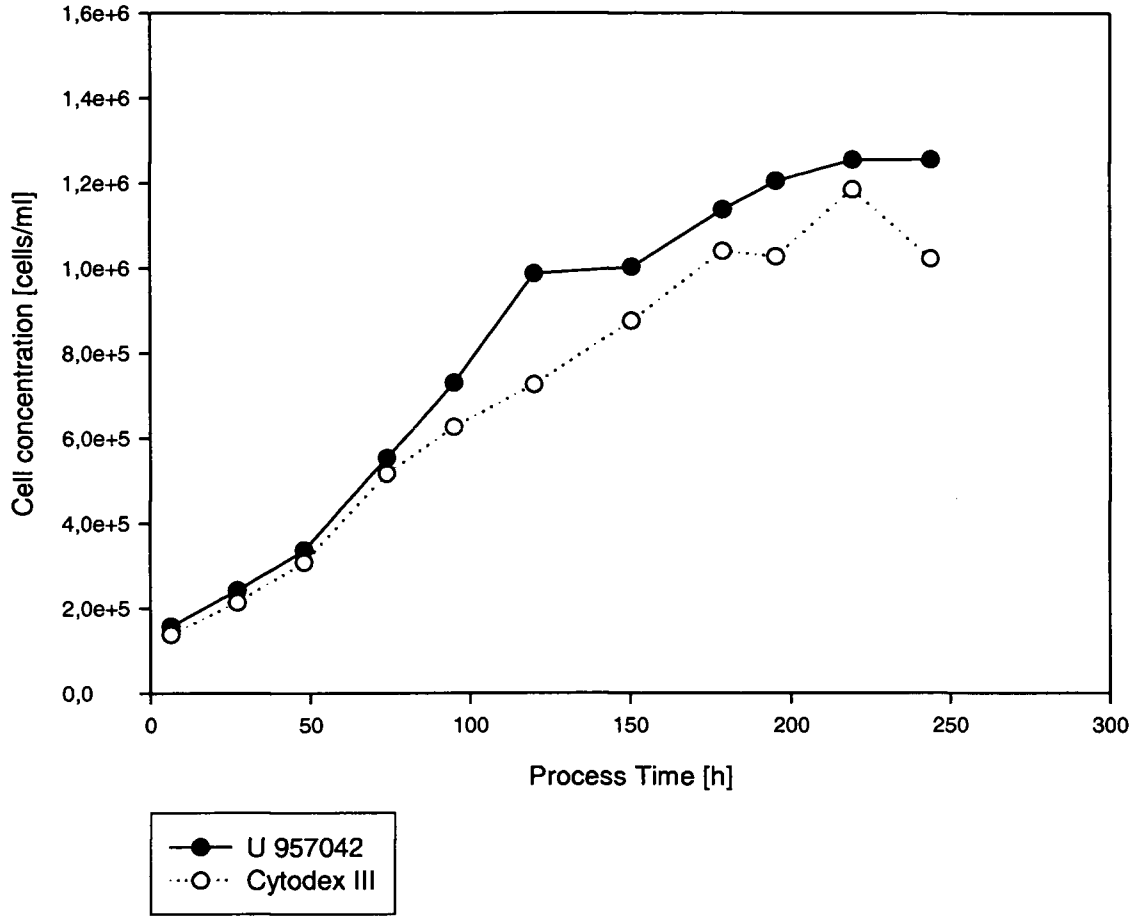


Figure 14: Cell growth on U957042

As shown in Figure 14 cell growth on recombinant collagen was comparable to Cytodex III or even slightly better. As the recombinant human collagen is far more expensive than the porcine one it was tried to coat Cytodex prototypes with reduced amounts of the protein. In a further experiment therefore 3 prototypes coated with 6, 9 and 44 mg recombinant collagen per g Sephadex G50 were compared to the 90 mg/g that had been used on U957042. However due to coating problems no cell growth occured on these prototypes. 5 more Cytodex modifications variing in the amount of coated collagen and crosslinker concentration were produced directly at Fuji TRL, the manufacturer of the recombinant collagen. In Figure 15 the growth curves of Vero cells on the tested prototypes 0409 MC 45A, 0409 MC 45B, 0409 MC 45D, 0410 MC 51A and 0410 MC 51C are shown. Except for day 4 the cell count on Cytodex III was always higher than on the test carriers. Their maximum cell concentrations reached 70 to 90% of Cytodex III.

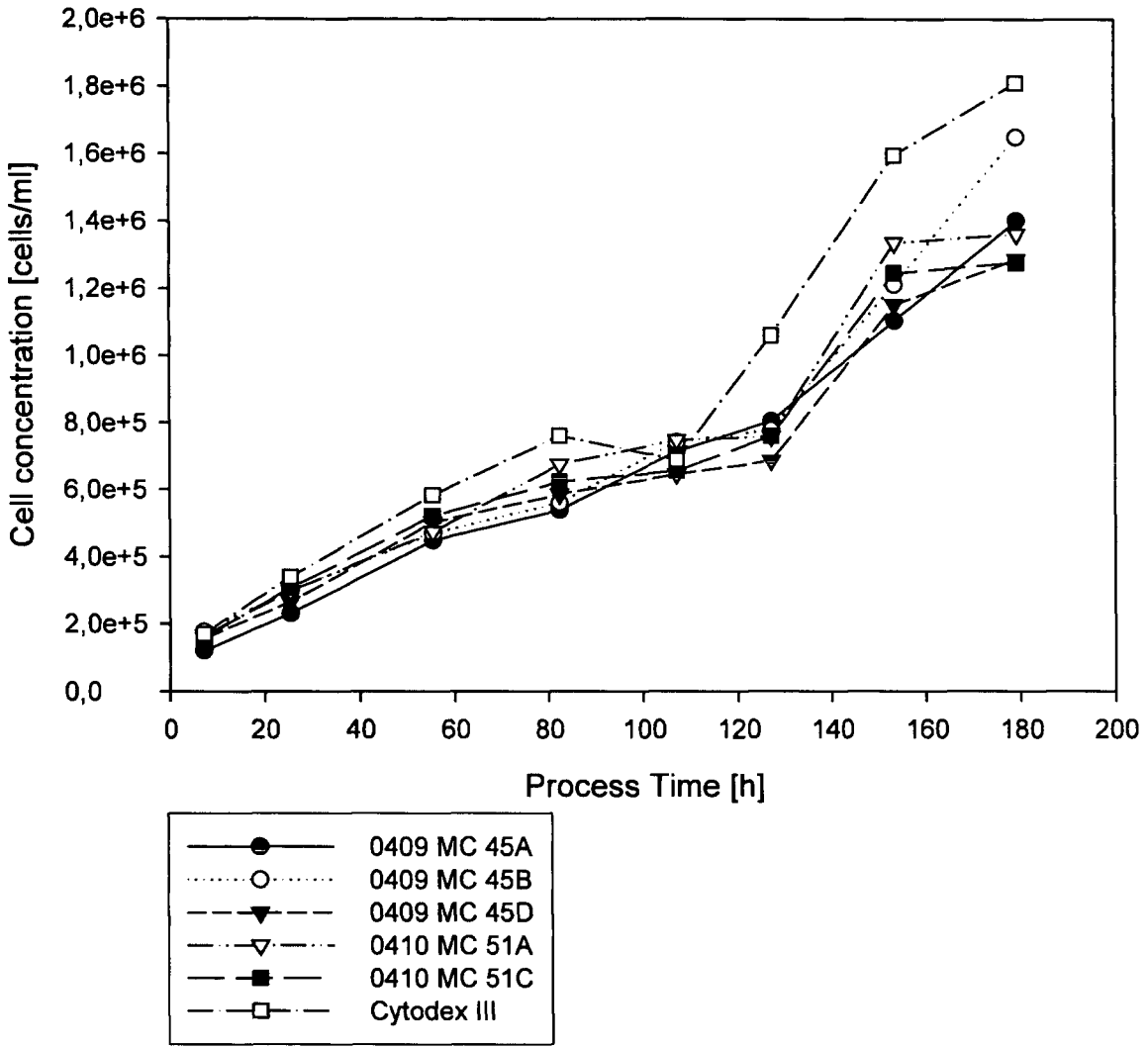


Figure 15: Growth curves collagen prototypes

6.1.3 A synthetic peptide as ligand

As lower cell growth occured on the prototypes coated with less collagen the use of recombinant collagen as coating material was given up due to the high costs. As alternative a synthetic RGDS (arginine-glycine-aspartic acid-serin) peptide was tried as ligand on the prototype U1309050. The results are shown in Figure 16. It should mimic a collagen coated surface as RGD is the core attachment motif in this protein. The carrier was tested together with an arginine prototype U1309051 having AGE as spacer and a ligand concentration of only 12 µmol/ml gel. As shown in Figure 16 the cell count on U1309050 was equal to Cytodex III and from 120 h on significantly higher than on the reference carrier. The maximum cell count was 170% of Cytodex III which in this experiment showed a lower cell count than Cytodex I. The maximum cell count on U109051 was 113% of Cytodex III. The reason for the superior performance of Cytodex I was that all carrier concentrations are calculated in g/l rather than cm^2/l because no surface areas for the prototypes are known. The surface area per gram carrier is 1.6 times higher for Cytodex I compared to Cytodex III. In most of the experiments the maximum cell

numbers were between 1.5 and $2.0*10^6$ cells/ml so the carriers were confluent and sometimes also had several cell layers on them but space was not limiting cell growth. In this case however the maximum cell count was $2.5*10^6$ for Cytodex III and $3.3*10^6$ for Cytodex I. As shown in Figure 16 the plateau phase that started after 168 h resulted in the lowest cell concentration for Cytodex III and most likely occurred due to lack of available space. The growth curve on U1309051 had a similar shape although at a slightly higher level with a maximum cell count of $2.7*10^6$ cells/ml. Cytodex I also showed a plateau phase starting on day 7 but due to the larger available surface the maximum cell number was higher. Interestingly the cell count on U1309050 did not level off so drastically. Microscopic observation however showed that the higher cell count was achieved by formation carrier aggregates that allowed the cells to grow on the additional space formed by cell bridging (see Figure 17).

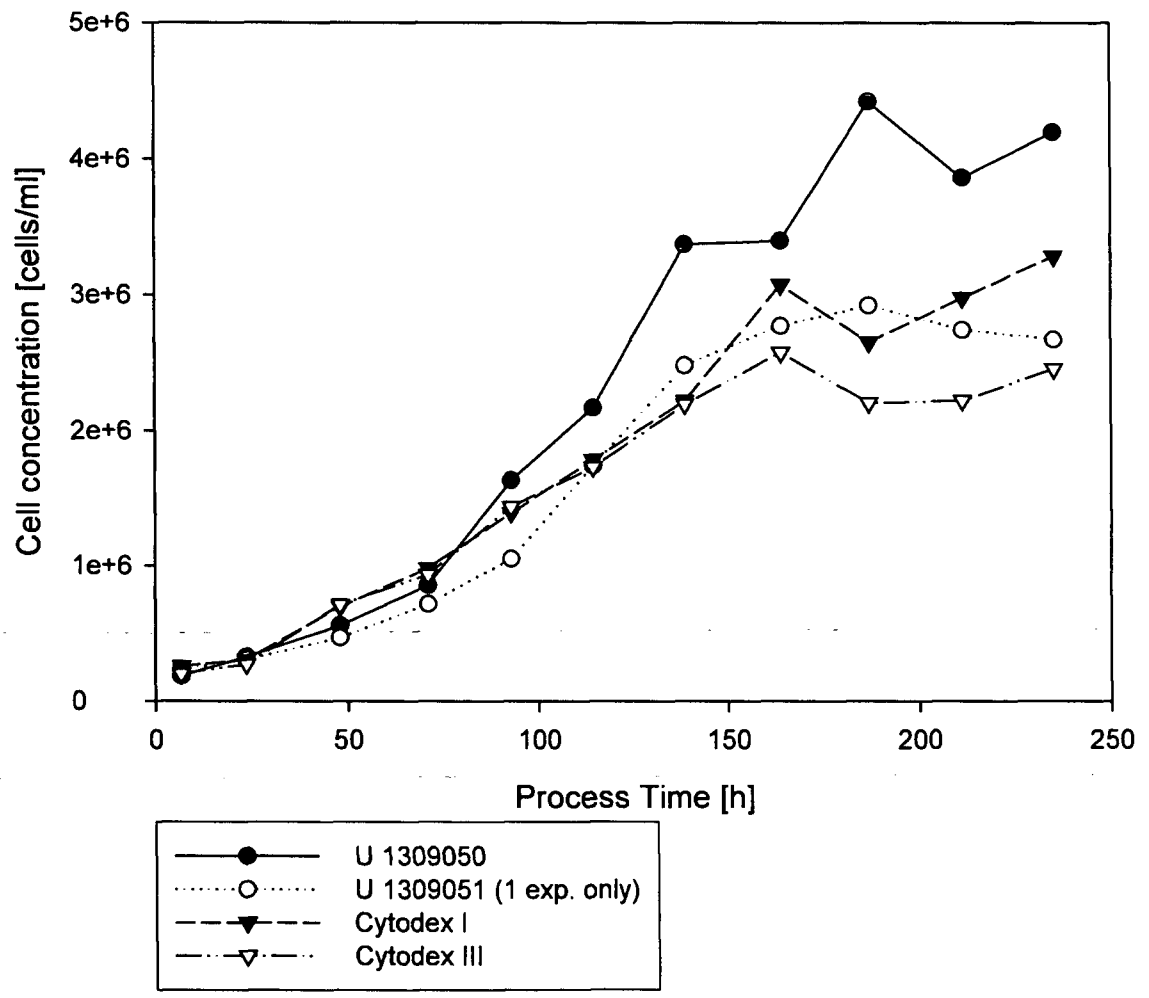


Figure 16: Cell growth on U1309050 and U1309051

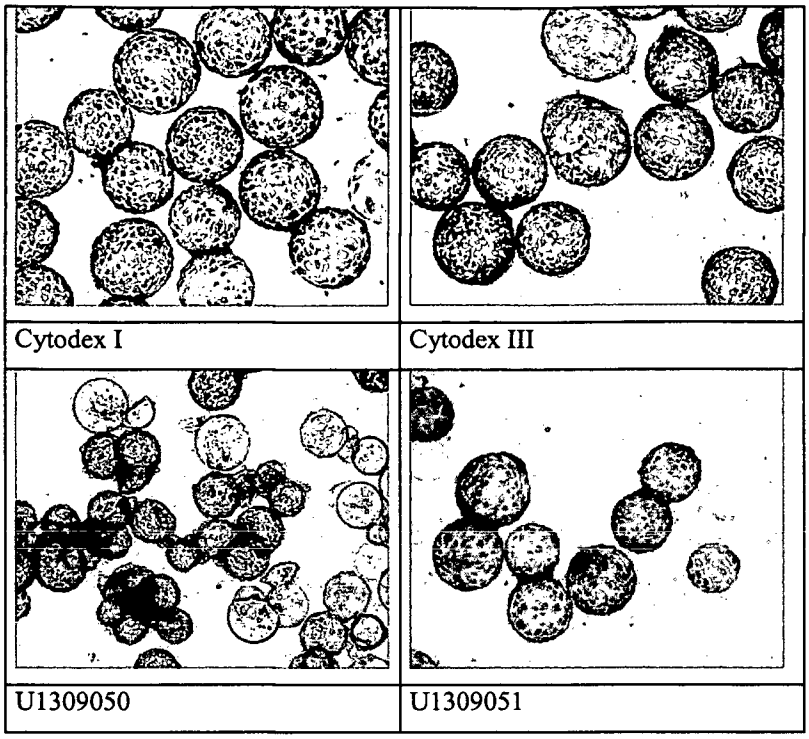


Figure 17: Cell growth on U1309050 and U1309051 after 115 h process time

6.1.4 Experiments with Sephadex G25

In this experiment 5 prototypes with a different matrix were tested. Sephadex G25 instead of Sephadex G50 was used. Sephadex G25 has a lower swelling volume which is roughly half the one of sephadex G50. It allows the coupling of more ligand to the same surface area compared to sephadex G50 and hence a higher surface charge concentration. The aim of the experiment was to test if a higher ligand concentration facilitates cell growth. In Table 9 it is shown that the medium ligand concentration is higher when Sephadex G25 is used. The maximum ligand concentration of the prototypes is 100 µmol/ml gel which is about 30% higher than the maximum concentration achieved with Sephadex G50. However the remaining 4 prototypes only have ligand concentrations in the range between 43 and 76 µmol/ml gel. Due to the reduced swelling volume also the density of the matrix is increased and it was more difficult to keep the carriers in suspension. The stirring speed had to be increased from 35 to 60 rpm for the first two days and finally to 75 rpm. The inflicted shear stress, however did not negatively influence cell growth as the performance of the reference carriers was normal (see Figure 18).

None of the prototypes showed a performance comparable to the reference carriers. As shown in Figure 18 only on U957077 an increase in cell number occurred after a two day lag phase. Nonetheless after 3 days of cultivation the cell count on this carrier reached only 43% of Cytodex III. No cell growth occured on the remaining 4 prototypes U957062, U957073, U957076 and U957080 which were below their inoculation cell count after 72 h process time.

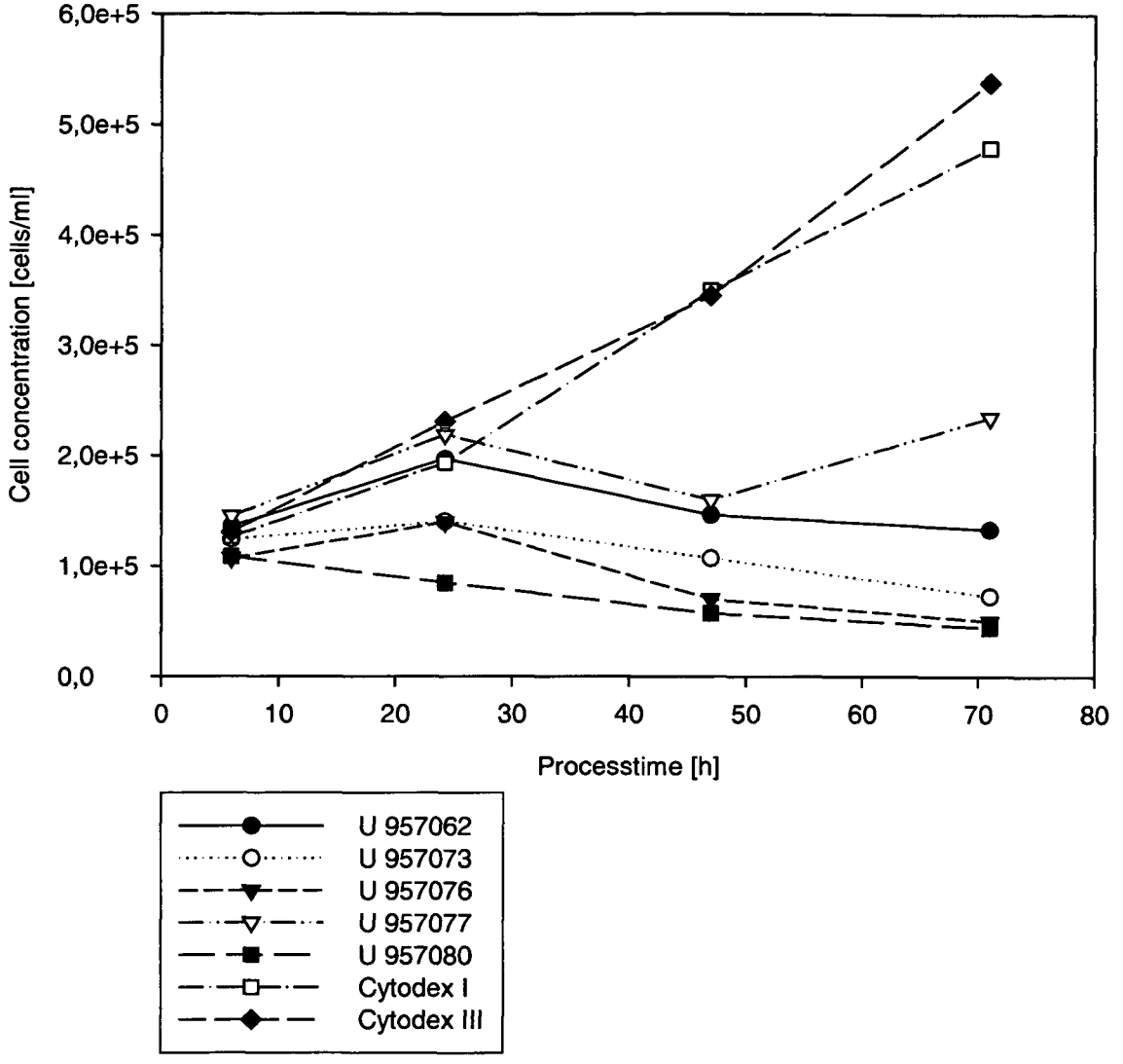


Figure 18: Growth curves Cytodex prototypes based on Sephadex G25

Carrier	Spacer	Ligand
	[µmol/ml Gel]	[µmol/ml Gel]
U957062	125	76
U957073	147	74
U957076	147	43
U957077	147	100
U957080	210	63

Table 9: Spacer and ligand concentration on sephadex G25 prototypes

6.1.5 Tests with arginine at varying spacer and ligand concentrations

12 samples of Cytodex Arginine Carriers were tested. The previous experiments had not shown a clear preference of the cells for high or low ligand concentrations. There were two arginine/allyl carriers that had a better performance than Cyotdex III. U957012 which had a ligand concentration of 36 µmol/ml gel and U1309051 with a ligand concentration of 12 µmol/ml gel. Both prototypes had a spacer concentration of 129 µmol/ml gel. The aim of the experiment was to get a better insight in the effect of different spacer and ligand concentrations on cell growth. For the 12 tested prototypes it was tried to keep both variables as independent as possible and to get high, medium and low spacer and ligand combinations, respectively. As shown in Table 10 there are combinations of high spacer concentration with high, medium and low ligand concentration for medium and low spacer concentrations obviously only medium and low ligand concentrations were possible. The tested spacer concentrations are between 125 and 185 µmol/ml gel, the tested ligand concentrations between 20 and 71 µmol/ml gel. The 12 prototypes were tested in 2 series (6 test carriers and Cytodex I and III reference carriers).

Carrier	Spacer. [µmol/ml Gel]	Ligand [µmol/ml Gel]
U1309075	185	71
U1309076	185	20
U1309077	185	53
U1309078	185	44
U1309079	146	42
U1309080	125	40
U1309082	186	57
U1309085	146	24
U1309086	125	29
U1309087	125	44
U1309089	185	39
U1309092	125	46

Table 10: Spacer and ligand concentration of arginine prototypes

As shown in Figure 19 none of the carriers tested in the first run showed a performance comparable to the reference carriers. The only prototype that supported cell growth was U1309075 after 48 h of process time however, cell count on this carrier was only 43% of Cytodex III. On the remaining carriers U1309076, U1309077, U1309078, U1309079 and U1309080 the cell counts had dropped below inoculation concentration therefore the experiment was terminated after 48h.

The results of the second run were more promising. Three of the tested prototypes supported cell growth as can be seen in Figure 20. Cells on these test carriers had a longer lag phase than on the reference carriers. After 48 hours they had between 50 and 70% of the cell count on Cytodex III. From then on cell growth accelerated especially on U1309082 which was equal to Cytodex III at 96 h and had slightly more cells (107 %) at 120 h. The two other carriers that supporedt cell growth were U1309092 and U1309087. At 120 h they have a cell count of

75 and 60% of Cytodex III. After 144 h the cultures entered a plateau phase. Cells on Cytodex III reached their maximum of $2.09*10^6$ cells/ml after 168 h in culture. The cultures with U1309082 and U1309092 reached their maximum cell numbers 24 h later with a relative cell count of 107 and 96 % compared to Cytodex III. The performance of U1309087 remained clearly below Cytodex III and also slightly below Cytodex I throughout the cultivation. The maximum cell number was 82% of Cytodex III.

As shown in Figure 20 the prototypes U1309085, U1309086 and U1309089 did not allow cell growth. After 48 h most of the cells on these carriers were detached and the experiments were terminated.

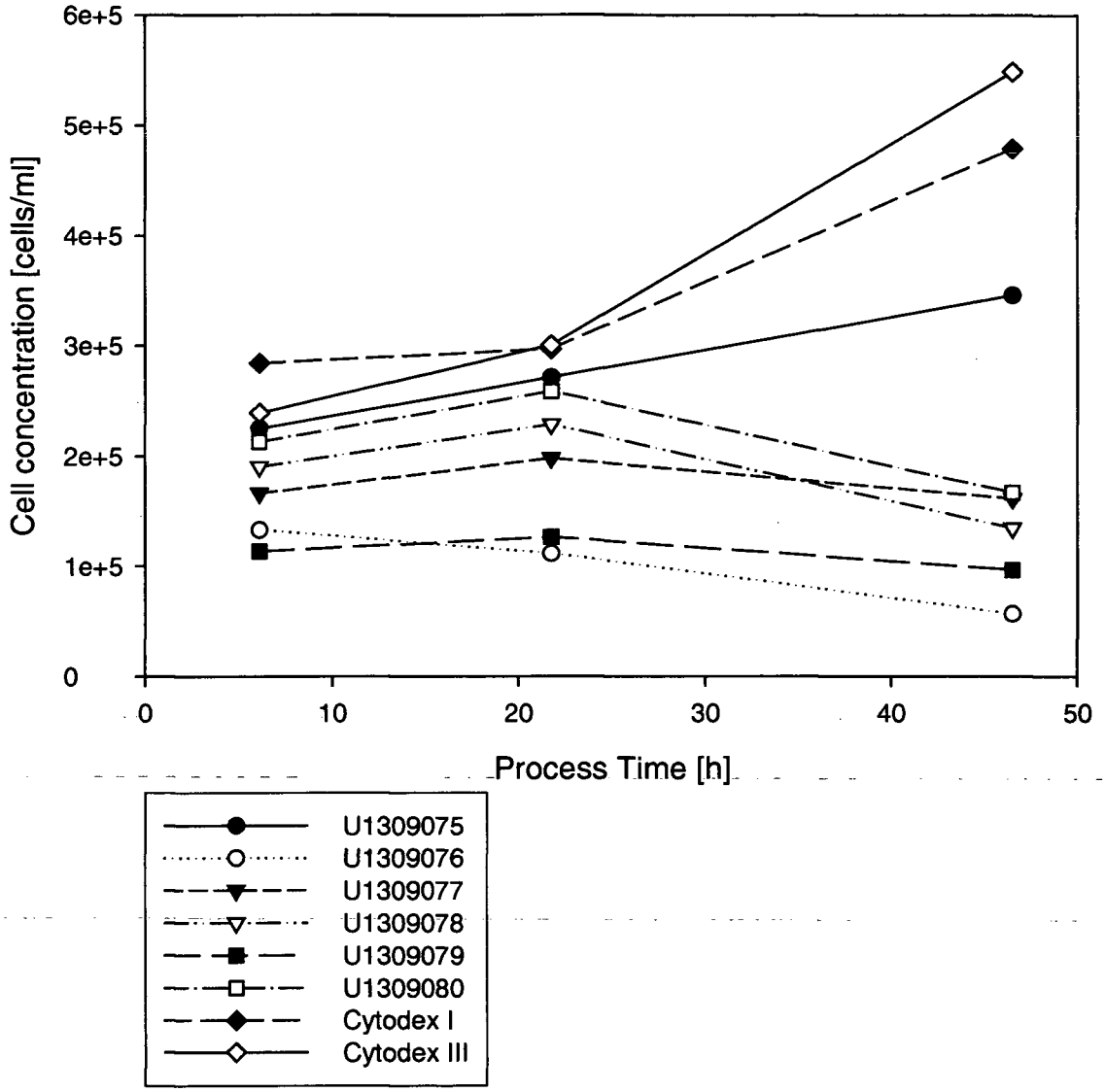


Figure 19: Growth curves for arginine based Cytodex prototypes

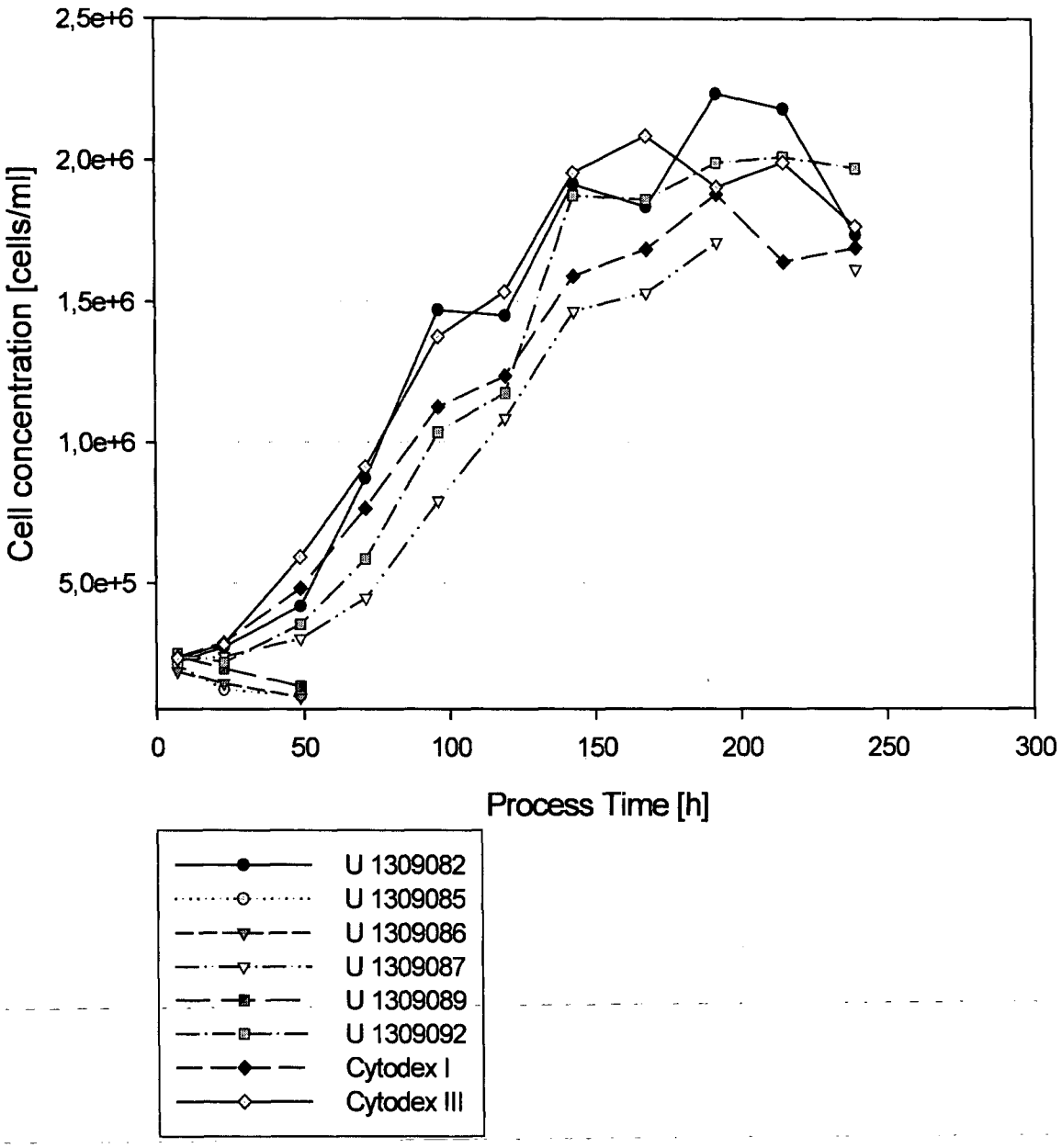


Figure 20: Growth curves arginine based prototypes

The 4 carriers that supported cell growth at least to some degree were U1309075, U1309082, U1309087 and U1309092. In Table 10 it can be seen that the first two carriers have a high spacer concentration of 185 µmol/ml gel and a high ligand concentration of 71 and 57 µmol/ml gel respectively. The latter two carriers have a low spacer concentration of 125 µmol/ml gel and a medium ligand concentration of 44 and 46 µmol/ml gel.

From the 4 carriers U1309082 has a performance equal to Cytodex III. Also U1309092 reaches a maximum cell number comparable to the reference carrier but only after 5 days in culture. U1309087 still reaches 82% while U1309075 only comes to 43% of the maximum cell count on Cytodex III. These results are shown in a 3D plot in Figure 21. If only the data from the 4 prototypes that supported cell growth is taken into account the conclusi-

on would be that spacer concentration is irrelevant and the optimum ligand concentration is probably in the range between 45 and 60 µmol/ml gel. However, this is not supported by the analysis data for the remaining carriers. From 12 prototypes 8 did not support cell growth. Four of these had ligand concentrations between 40 and 60 µmol/ml gel. The specifications of U1309082 the best working carrier of the experiment with a spacer concentration of 186 µmol/ml gel and a ligand concentration of 57 µmol/ml gel are practically the same as of U1309077 (spacer concentration 185, ligand concentration 53µmol/ml gel) but cells do not grow on this carrier.

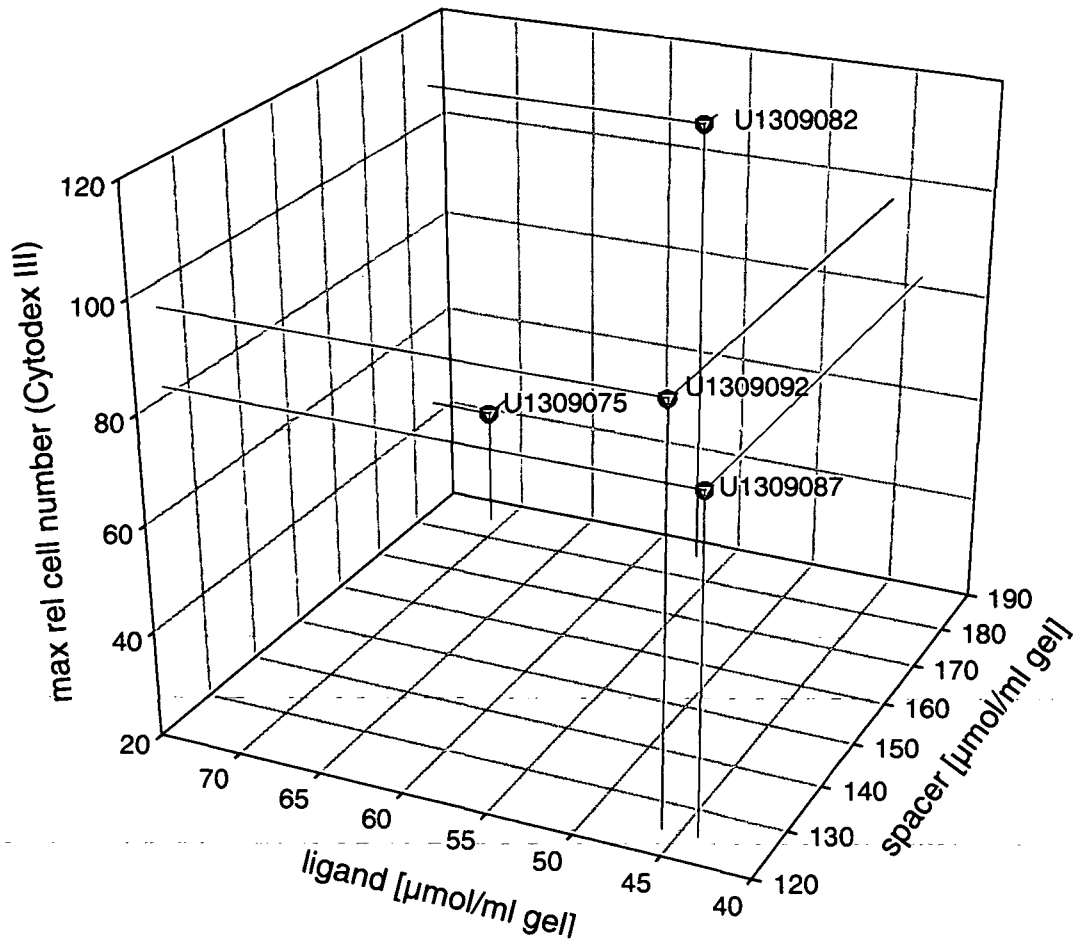


Figure 21: Maximum cell numbers on selected Cytodex-Arginine prototypes

6.1.6 Downscale of the carrier tests

Testing carrier prototypes in spinner flask experiments gives quantitative data about cell growth but takes up a lot of time. In an effort to develop a screening system carrier tests were conducted in microwell plates. The aim was to get a tradeoff between throughput and accuracy.

In a first series of experiments Cytodex I, Cytodex III, sephadex G50 and allylated sephadex G50 (i.e. matrix and spacer only) were immobilised on petri dishes. The polystyrene material of the dishes was softened by acetone treatment, the carriers were added and covered with glycerol to prevent desiccation. Before inoculation the dishes were washed with PBS and medium. As polystyrene is not autoclavable Vero cells were cultivated in medium supplemented with penicillin and streptomycin. The experiment was designed to give a qualitative result if cells attached and grew on the immobilised microcarriers. Cell growth was veryfied by microscopic observation. The time points were the same as used for the experiments in the spinner flasks namely 6 and 24 h after inoculation.

As shown in Figure 22 the immobilisation did not work equally well for the different carriers. For Cytodex I and III a rather homogeneous distribution was achieved while only few sephadex G50 carriers were attached to the petri dish. The allylated sephadex carriers have a polygonal instead of the usual round form. Cells did not attach to sephadex G50 and allylated sephadex however Figure 22 clearly shows that cell attachment occurred on Cytodex I and III. Also cell morphology does not seem to be altered as the cells on the immobilised Cytodex III look similar to the ones grown on suspended carriers as shown in Figure 17.

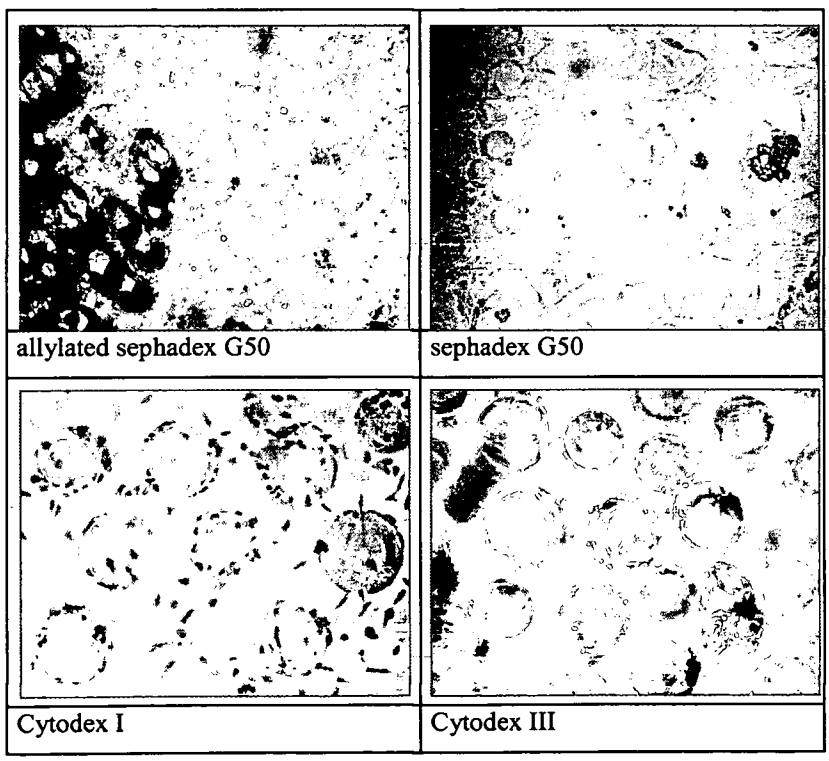


Figure 22: Cell attachment on Cytodex carriers immobilised on petri dishes (6h after inoculation)

Cultivation of Vero cells on immobilised carriers gave promising results. In order to increase value and comparability of the results it was tried to switch to a system that would allow to measure cell growth in 96 well plates. The general cell screening system (GCSS) uses specially designed 96 well plates to measure cell growth by the reduction of transmitted light and growth curves for every individual well can be obtained.

3 lines of a 96 well plate (line F-H) were coated with different concentrations of Cytodex III while 2 lines (line A and B) contained suspended Cytodex III to test if the immobilisation method disturbed the measurement. The immobilised carriers were washed two times with PBS and then incubated over night with medium containing

Penicillin and Streptomycin. For the GCSS plate blank values were determined before the addition of the Vero cells. Two Inoculum concentrations were tested. For the columns 1 to 6 $2*10^5$ cells/ml and for columns 7 to 12 $4*10^5$ cells/ml were used. The working volume was 150 µl per well. The plates were incubated at 37°C in an atmosphere containing 7% CO2. To determine cell attachment microphotographs were taken 2 hours after inoculation. At the same time also light transmission of the inoculated wells in the GCSS plate was measured. These analyses where repeated daily. In total the cells were cultivated for 6 days with a 50% medium change after 4 days.

Two hours after inoculation the cells were still rounded but had attached to all of the carriers. There were no visible differences between cells on the immobilised and on the settled carriers. A lot of cells, however attached to the plate, probably because the plate was plasma treated and the carriers did not fully cover the plastic. After 24 hours the cells had completely spread out. During growth phase no significant differences in cell morphology on settled and immobilised carriers were noted. The settled carriers however showed a more uniform cell distribution whereas on the immobilised carriers the cells showed a tendency to form annular aggregates possibly in the crevice between plate material and the carrier as shown in Figure 23. Cell growth also seemed to progress faster on the settled carriers. If the cell distribution after 72 hours is compared it can be noted that on the settled carriers the cells are already reaching confluence while on the immobilised ones there is no closed layer. After 144 h however the cells on the immobilised carriers caught up and more or less covered the whole carrier.

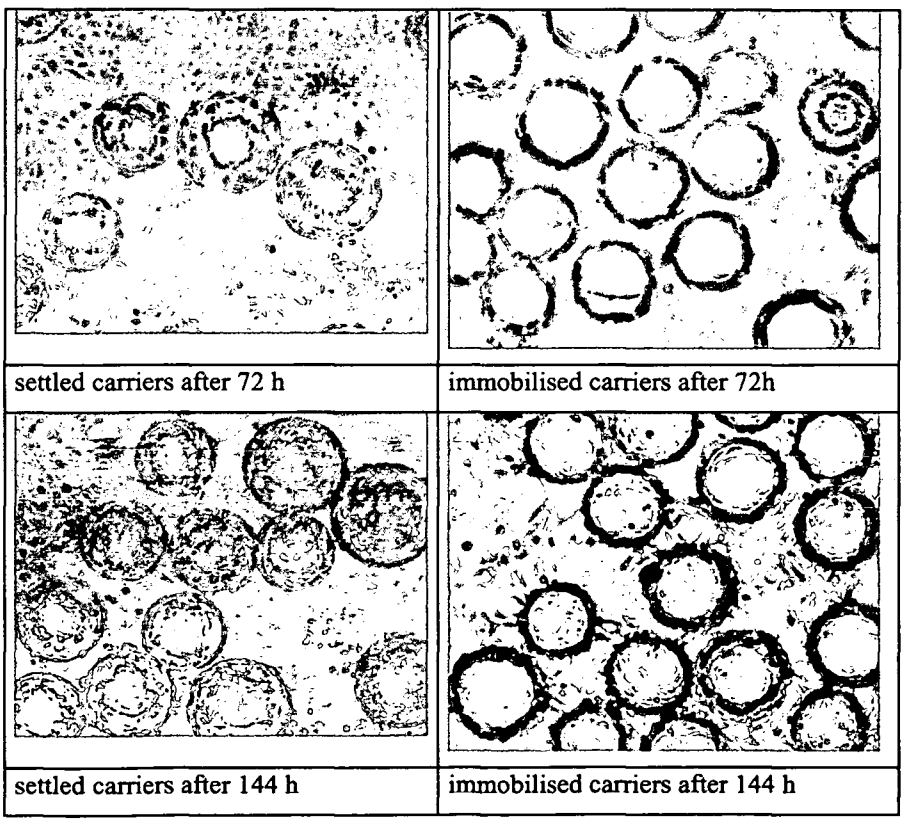


Figure 23: Cell growth on settled and immobilised Cytodex III

Absorption measured in the GCSS increased parallel with microscopically observed cell growth . Starting from the third day of cultivation the readings were significantly higher than the background. The recorded growth

curves of the GCSS plate are shown in Figure 24. The rows C, D and E are empty and the high values are artefacts due to condensate formation on the lid. A zoomed curve of a single well is shown in Figure 25. The influence of inoculum concentration on cell growth was clearly visible. For the columns 7-12 the higher one had been used. The cells reached a nutrient limit earlier and wer already in the stationary phase in most of the wells. In some cases they did not seem to enter the exponential growth phase on the immobilised carriers. The situation was better for the lower inoculum concentration (columns 1-6) where the cell growth on suspended and immobilised carriers was more comparable and all cells were in the exponential phase.

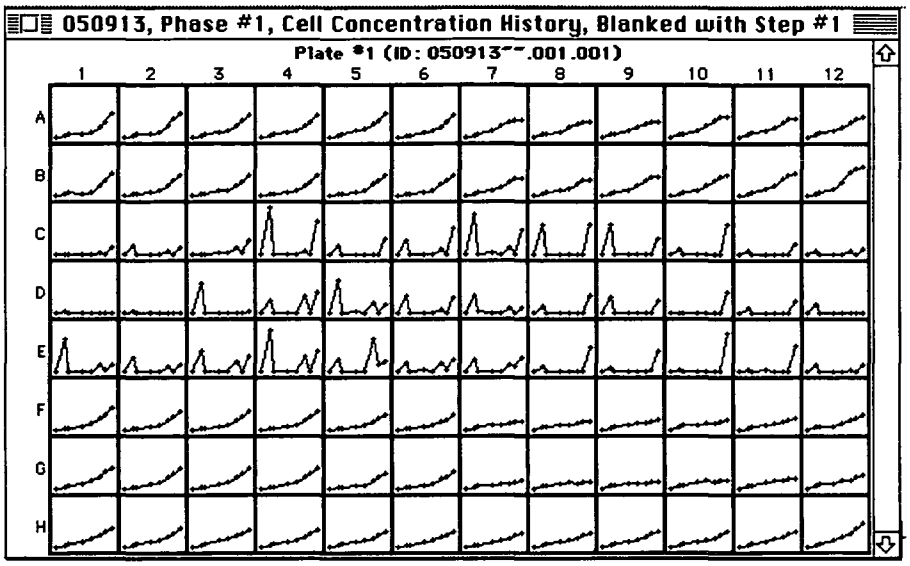


Figure 24: Growth curves on Cytodex III according to GCSS measurement

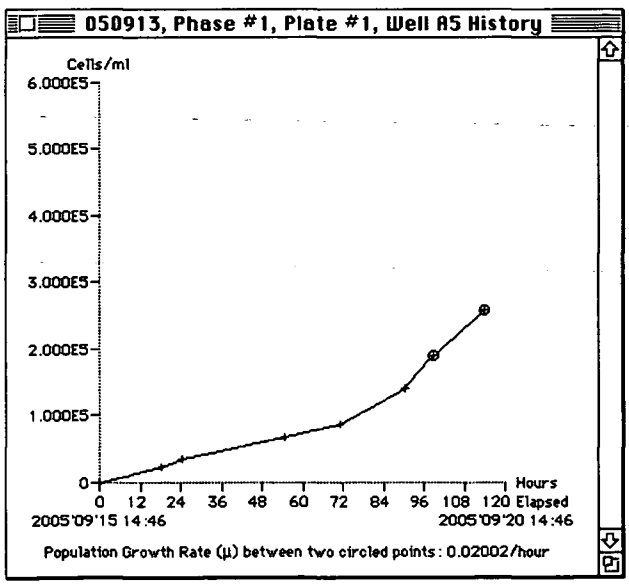


Figure 25: GCSS growth curve of a single well

Vero cells grew on immobilised as well as on settled Cytodex III and cell morphology was comparable on both carrier types. While cell growth was slower on the immobilised carriers there were in both cases confluent carriers after 6 days process time. Cell growth as measured by GCSS correlated well with microscopic observation.

There was significant cell growth on the plate material not covered by carriers. These cells of course influenced the GCSS readings however the problem may be overcome by coating more carriers per well in order to achieve a monolayer and reduce the plastic area available to cell growth. An increase in carrier concentration would furthermore allow higher cell numbers to be cultivated per well. In this case media changes would be necessary to prevent nutrient limitation however the higher cell concentration would further increase accuracy of the GCSS measurement. On the immobilised carriers the cells form annular aggregates presumably in the crevice between carrier and plate. During the immobilisation procedure the plate material is softened by acetone treatment and the carriers are embedded in it. As the formation of cell aggregates is greatly reduced when the cells are grown on settled carriers it seems favourable to use rather mild immobilisation and prevent the carriers from being trapped in the plastic too deeply.

After these preliminary tests 48 Cytodex prototypes were tested in static systems either in GCSS plates or different kinds of microtiterplates. An overview of their chemical properties is given in Table 8. The ligands most widely used were arginine and DEAE. On 23 of the prototypes arginine was used and 17 had DEAE as ligand. Additionally 8 carriers with lysine, agmatine or arginine derivatives were included in the experiments.

To test the suitability of the GCSS for recording cell growth on other Carriers than Cytodex I and III seven different Cytodex modifications were tested in spinner flasks and as immobilised and settled carriers in GCSS plates. DEAE was tested as ligand in different coupling methods with dextrane and PVA as spacer. The carrier specifications are shown in Table 11. Cytodex III was used as reference carrier.

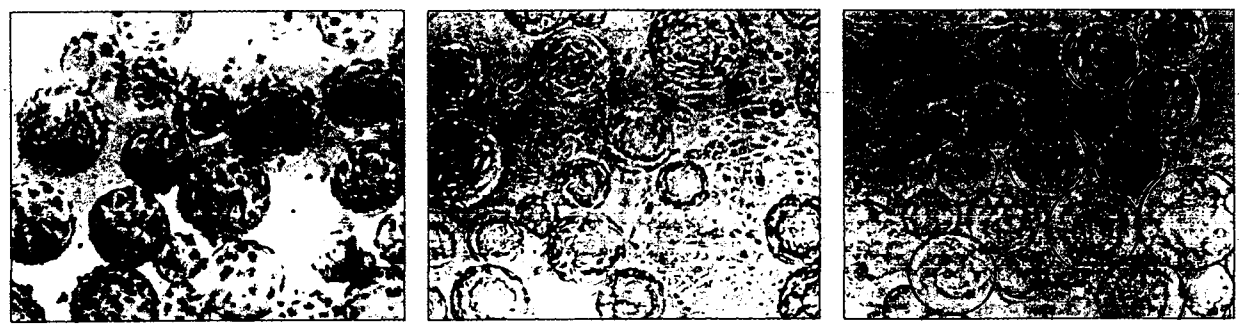
Carrier	Matrix	Spacer	Conc. [µg/ml]	Ligand	Conc. [µg/ml]
U1453040	Sephadex G50	Dx 40k	35.7	DEAE	43
U1453045	Sephadex G50	Dx 40k	36.7	DEAE	51
U1453048	Sephadex G50	Dx 40k	44	DEAE	75
U1453050	Sephadex G50	PVA	n.a.	DEAE	26
U1453051	Sephadex G50	PVA	n.a.	DEAE	40
U1453052	Sephadex G50	DEAE-Dx 500k	n.a.	--	45
U1453054	Sephadex G50	DEAE-Dx 500k	n.a.	--	68

Table 11: Specifications of DEAE prototypes

The experiments in the spinner flasks were carried out as previously. For the experiments in the GCSS plates antibiotics (Penicillin and Streptomycin) were added to the culture medium. The GCSS plates (2 replicates per carrier concentration) were coated with the seven prototypes and Cytodex III.
Inoculum concentration was $2.4*10^5$ cells/ml for the spinner flasks and $7*10^4$ cells/ml for the GCSS plates. To assess cell morphology and growth microphotographs were taken 7 h after inoculation and then daily. Cell counts were performed on samples removed from the spinner flasks.

Seven hours after inoculation the cells on the immobilised carriers were still rounded except for the ones on Cytodex III. On the settled carriers there were more differences visible. Cells on U1453048 and U1453051 started to flatten out, on Cytodex I and III they were already tightly attached while on the negative controls (Sephadex G50 and allylated Sephadex G50) no cells were visible. Cell morphology in the spinner flasks was the same as on the settled carriers. After 2 days also the cells on the immobilised carriers U1453048 and U1453051 were tightly attached. From then on there were only minor differences between the 3 cultivation systems. For U1453051 and 3 days of cultivation this is shown in Figure 26.
It was also tried to record cell growth on the GCSS plates by the increase in absorption. Unfortunately growth curves looked the same for all prototypes. As shown in Figure 29 the bottom of the wells was in no case completely covered with microcarriers (although the Cytodex III wells looked better than the rest). Therefore a lot of cells grew on the plastic. Presumably they produced such a high background that the different cell growth on the microcarriers could not be distinguished.

Microscopic observation and cell count showed that cells only grew on the carriers U1453048 and U1453051 so the spinner experiments with the other 5 prototypes were stopped after 4 days. Although the carriers U1455040 and U1455045 showed rather good attachment characteristics cells did not grow on them but remained rounded and were eventually shed off in the spinner flasks. Cells on the prototypes U1453048 and U1453051 reached about the same cell count as on the reference carriers. The growth curves are shown in Figure 27. As judged by microscopic observation the cells on U1453051 were more flattened and also more homogeneously distributed than on U1453048. After the 120 h of cultivation this difference was no longer visible. Also by comparison of the relative cell counts 6 h after inoculation as well as at 72 and 192 h of the cultivation it becomes evident that the cells on U1453048 eventually caught up (see Figure 28).



cultivated in spinner flask | cultivated on settled carriers | cultivated on immobilised carriers

Figure 26: Cell morphology on U1453051 in different cultivation systems

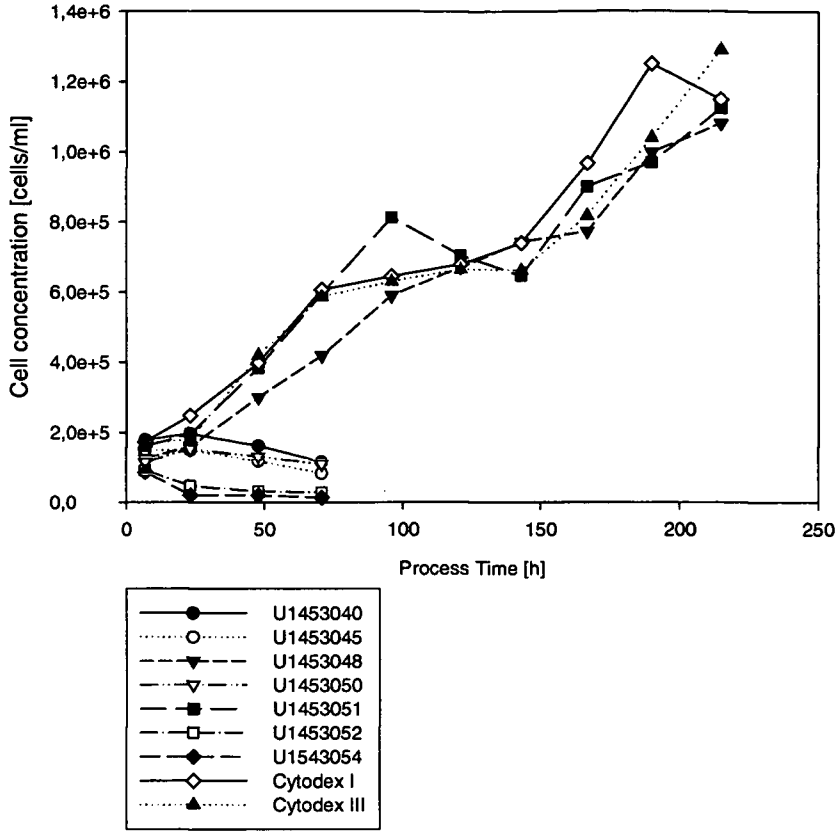


Figure 27: Cell growth on Cytodex DEAE prototypes

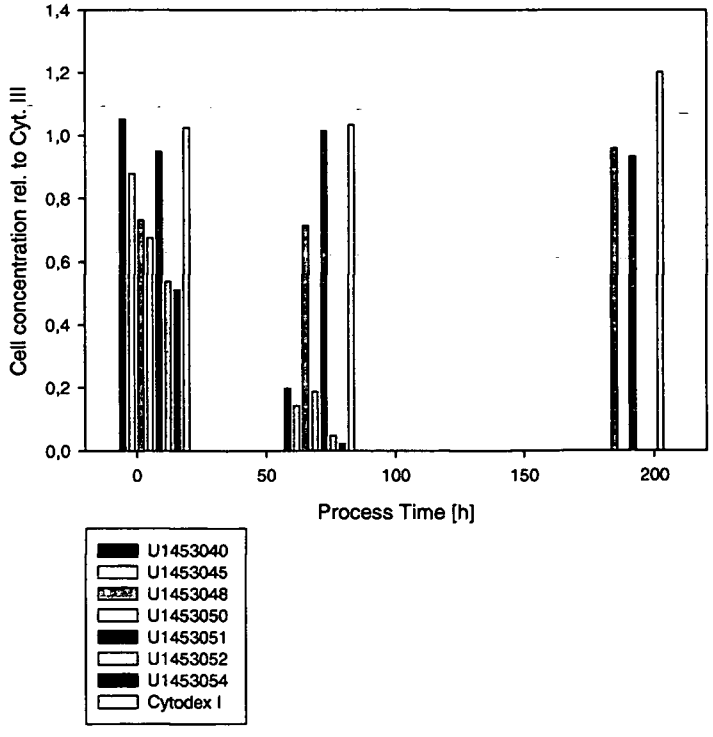
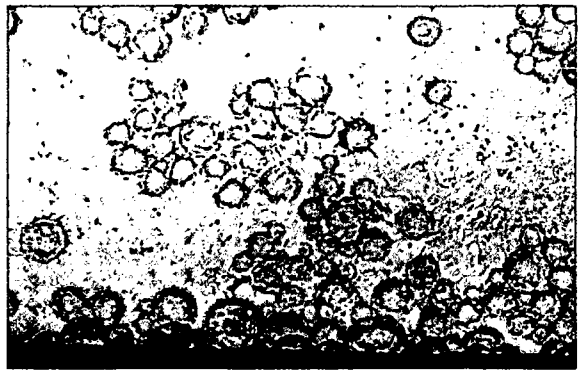
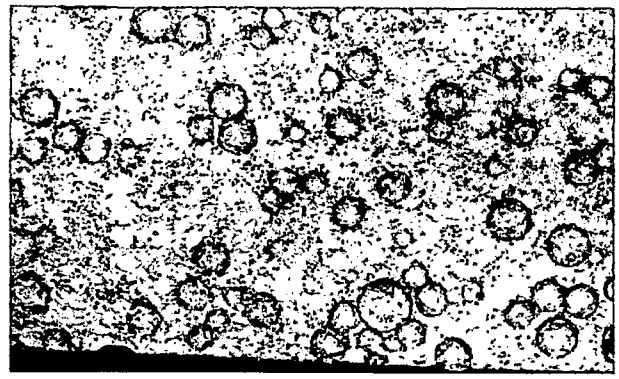


Figure 28: Cell count on Cytodex DEAE prototypes relative to Cytodex III



immobilised U1453048



settled U1453048

Figure 29: Carrier distribution in GCSS plates

In a further experiment it was again tried to use the GCSS for measurement of cell growth on 12 immobilised Cytodex modifications. All immobilised prototypes were also tested as settled carriers (see Table 12). Besides the prototypes Cytodex I and III were used as positive controls and Sephadex G50 as negative control. The inoculum concentration was $7*10^4$ cells/ml with a working volume of 150 µl/well. The cells were cultivated for 6 days with a 50% medium change after 4 days. To assess cell morphology microphotographs were taken 7h, 1 and 6 days after inoculation. GCSS measurements were done after 1, 4 and 6 days.

U1403040A	U1574045
U1403048A	U1453096
U1403048B	U957062
U1574018	U957077
U1574044	

Table 12: Prototypes tested in the second GCSS run

The quality of the GCSS results was impaired by coating problems. The amount and uniformity of coated carriers varied widely among the prototypes. The best coverage was obtained with Cytodex III. Second best was the coating with Cytodex I where the distribution of the carriers within the well was not as good. Among the prototypes the coating was good for U1403040A, U1403048A, U1403048B and U1575044 where the well bottom was not covered but there was at least a rather uniform distribution of the carriers. In all other cases there were either few carriers immobilised in the well or they were predominantly attached to the edges. Unfortunately the immobilisation did not work for Sephadex G50. This resulted in a large percentage of uncovered plastic in those wells. Cells therefore grew also in the wells which were meant to be the negative control. Although it was verified microscopically that almost no cells were attached to the carriers the high background disturbed GCSS measurement. Seven hours after inoculation only cells that had attached to the plastic of the microtiterplate and some cells attached to Cytodex III were spread out. On all other carriers including Cytodex I the cells were still rounded. The cell morphology seemed to be slightly better on the settled carriers than on the immobilised ones. One day after inoculation the cells were spread out on Cytodex I and III and there was no difference between settled and immobilised carriers. Cells on Cytodex I and III were confluent after 6 days.

Clearly positive results were only obtained for U1574045 as only on this Cytodex modification substantial cell growth on immobilised and settled carriers had taken place after 6 days however it was clearly below the one obtained for Cytodex III. On U1403048 cells grew only on the immobilised carriers on U1574044 cell growth only ocurred on the settled carriers. The prototypes U957062 and U957077 had a different matrix. They were darker and less transparent which makes it impossible to follow cell growth using the GCSS it was however verified microscopically that cells did not grow on these carriers.
The GCSS results from this screening were not in accordance with microscopic observation. The reason for this was mostly the inhomogeneous coating of the wells and the resulting cell growth on the unoccupied plastic surface. Due to the high background GCSS readings did not show significant differences for the various prototypes.

To adress the coating problem different immobilisation methods were tested in a further experiment. Cytodex I carriers were attached to the wells of two GCSS plates using 6 different methods as shown in Table 13. For the immobilisation in acetone treated wells 3 different concentrations of carriers were used as coating was done with 10, 20 and 50 µl of carrier slurry.
In the coating experiments on the plate U1403067 several wells were not transparent. The carriers that were immobilised in silicone did not swell, for the immobilisation experiments with Loctite glue too many carriers were used. After swelling the wells were almost completely filled with carriers. Consequently cell growth could neither be followed microscopically nor using the GCSS. Carriers immobilised with Toluene/Ethanol 1/4 formed a monolayer but did not swell. A Toluene/Ethanol mixture 1/1 or a Toluene/Hexane mixture 1/3 made the plastic intransparent. When immobilised using PBS/Acetone only few carriers were retained in the wells and no difference in the amount of attached carriers was observed in the wells coated with 10, 20 and 50µl carrier.
On plate U1076031 the carriers were immobilised using toluene. The treatment however resulted in intransparency of all wells.

U1403067	U107631
Cytodex I immobilised in Silicone	Cytodex I immobilised using Toluene
Cytodex I immobilised in Loctite	
Cytodex I immobilised using Toluene/Ethanol	
Cytodex I immobilised using Hexane	
Cytodex I immobilised using PBS/Acetone	

Table 13: Immobilisation methods tested for Cytodex I

In addition to the coating experiment with Cytodex I, eight prototypes immobilised by acetone tretament were tested their specifications are shown in Table 14. Cytodex I and III were used as reference carriers.
The amount and uniformity of coated carriers varied widely among the prototypes. The best coverage was obtained for U1574054 and U1574056. Interestingly these carriers were more closely packed than Cytodex III. Nevertheless the bottom was not fully covered and there were some spaces where cell growth occured on the plastic. Only few carriers were attached in the case of U1574055, U1574062 and U1574065. In no case a mono-

layer of carriers covering the whole well bottom was achieved. The resulting background due to cells growing on the plastic makes the use of the GCSS very error prone. In wells with less than 50 to 100 carriers even judgement of cell growth by microscopic observation is inaccurate. A representative picture of such a situation is shown in Figure 30.

Carrier	Spacer	Ligand	Matrix
	[µM/ml Gel]	[µM/ml Gel]	
U784091	Allyl, 191	Arg, 31	Sephadex G50
U957002	Allyl, 191	Arg, 27	Sephadex G50
U1574054	none	DEAE, 27,5	Sephadex G50
U1574055	none	DEAE, 50	Sephadex G50
U1574056	none	DEAE, 24	Sephadex G50
U1574062	DX 40, 11	DEAE, 115	Sepharose 4FF
U1574063	Allyl, 318	Arg, 70	Sephadex G50
U1574065	none	DEAE, 39,5	Sepharose 4FF

Table 14: Specifications of immobilised prototypes

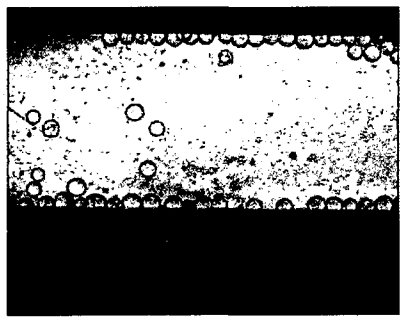


Figure 30: Cytodex I immobilised using Acetone

Six hours after inoculation cells were only spread out on Cytodex III. No cell attachment occurred on U1574063. On all other carriers including Cytodex I cells were still rounded. One day after inoculation cells began to spread out on Cytodex I. Cells on Cytodex I and III were confluent after 4 days. Concerning cell attachment none of the tested Cytodex modifications had a performance similar to Cytodex III. However U1574062 and U1574065 are promising. Cells on these carriers grew similar as on Cytodex I and were more or less confluent after 4 days as shown in Figure 32. On the other test-carriers (U784091, U957002, U1574054, U1574055, U1574056 and U1574063) the cells were still rounded after 4 days. The situation was the same 6 days after inoculation.
Due to the patchy coating and the resulting background the GCSS was not suitable to measure cell growth. As depicted in Figure 31 no significant difference can be seen in the growth (C5 and 8 are outliers).

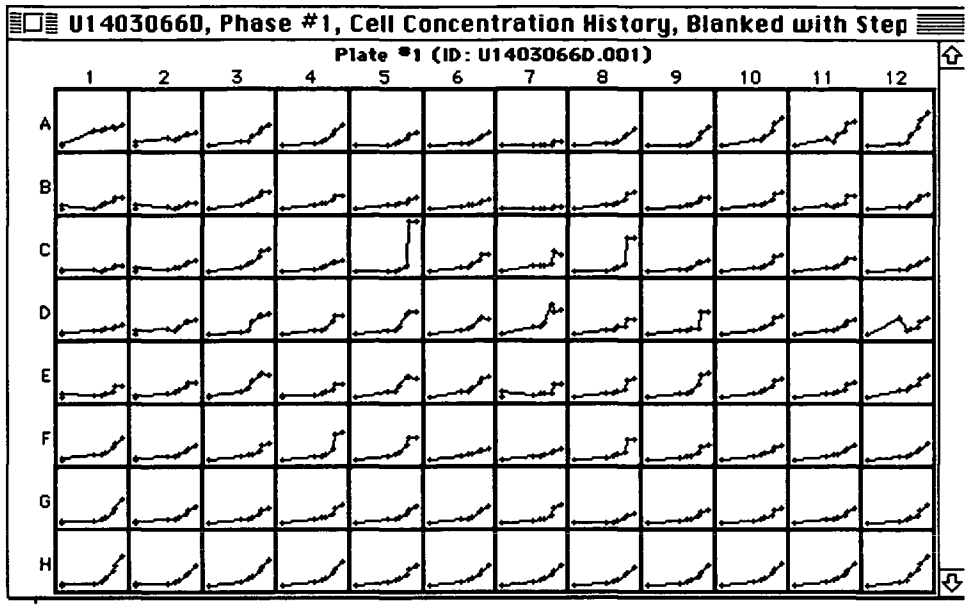


Figure 31: GCSS growth curves for immobilised prototypes

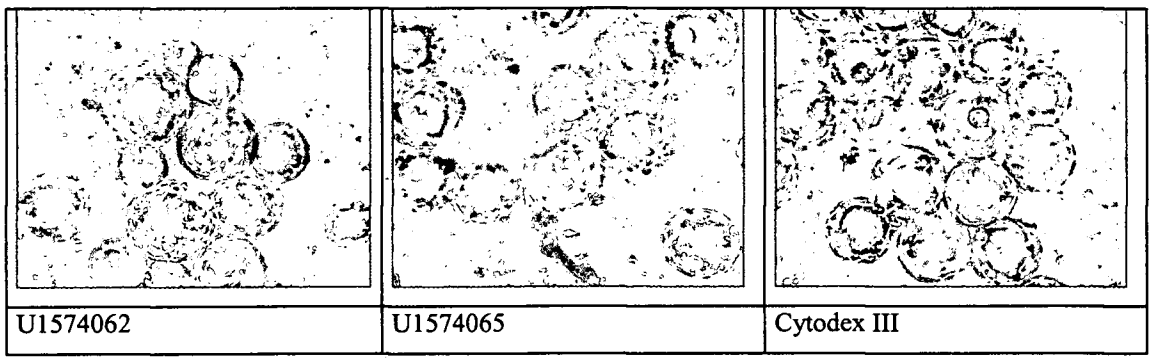


Figure 32: Cell growth on Cytodex III and the two best prototypes after 4 d

It turned out that the problems to get the well bottoms homogeneously covered with carrier could not be solved. The following experiments were therefore conducted on settled carriers in microtiterplates.

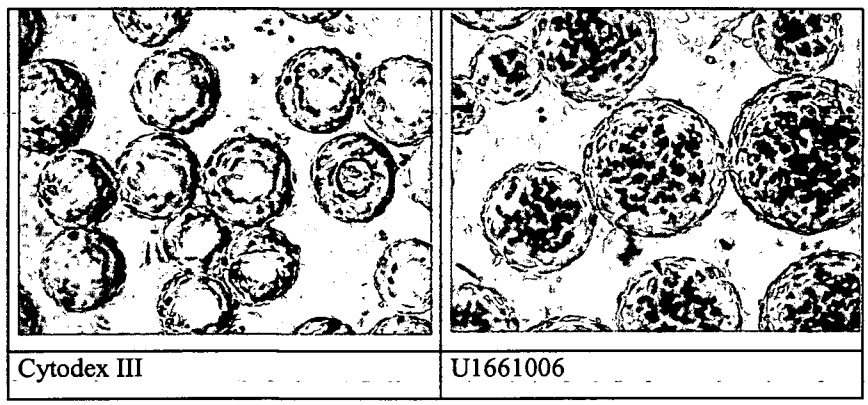
Vero cells were cultivated on 4 prototypes with Cytodex III as reference carrier as shown in Table 15. Two of the carriers U1661006 and U1661007 had magnetic particles included for easier carrier retention. U1574066 had a DEAE concentration of 110 µmol/ml gel and 42 µmol/ml gel Dx 40k as spacer on a Sephadex G50 matrix. U 1661003/1 was an arginine based carrier with a ligand concentration of 30 µmol/ml gel attached to a Sephadex G50 matrix via an epoxy group at a concentration of 63 µmol/ml gel. The test was performed in duplicates using 12 well plates with a working volume of 2 ml. To facilitate microscopic observation a carrier concentration of only 0.45 g/l was used. Under this conditions the settled carriers do not lie on top of each other but rather form a monolayer. Inoculum concentration was 1.3 E+05 cells/ml except for U1661003/1 which were inoculated with $2.3*10^5$ cells/ml as the beads are rather small and therefore most likely have a larger surface area. To assess cell morphology microphotographs were taken 6, 24, 48 and 96 hours after inoculation. On U1661007 and especially U1661006 the magnetic particles in the carriers impaired microscopic observation.

U1574066	U1661007
U1661003/1	Cytodex III
U1661006	

Table 15: Prototypes used for the experiments with settled microcarriers

Six hours after inoculation cells were only spread out on Cytodex III, however cells also started spreading on U1661006. Whereas the cells had attached to the other test carriers but remained rounded.

After 24 hours the Vero cells were also spread on U1661006 while they remained rounded on the other test carriers. Interestingly, cells started to aggregate around the magnetic particles of U1661007 but nonetheless kept their rounded morphology. As shown in Figure 33 cells reached confluence on Cytodex III and U1661006 after 48 hours. No morphological change was noted on the other test carriers. The experiment was stopped after 4 days. At this point cells were still confluent on Cytodex III and U1661006 on these carriers even an additional cell layer started to form. On U1661007 cells began to grow starting from the mentioned aggregates however this growth is most probably due to conditioning of the carrier surface by extracellular matrix proteins secreted from the aggregated cells. On U1574066 and U1661003/1 cells were still rounded after 4 days and no cell growth was noticed.



Cytodex III | U1661006

Figure 33: U166006 and reference after 48 h cultivation

Tests in 12 well plates were then run for 20 prototypes that were combined of seven different spacers, six different ligands and three different matrices. The carrier specifications are shown in Table 16. To assess cell morphology microphotographs were taken 7, 24 and 48 after inoculation.

Carrier	Spacer	Spacer [µmol/ml gel]	Ligand	Ligand [µmol/ml gel]	Matrix
U1689080A	Epoxy	n.a.	Arg	58	Seph 6FF
U1689080B	Epoxy	n.a.	Arg	103	Seph 6FF
U1689080C	Epoxy-Allyl Dextran	80	Arg	33	Seph 6FF
U1689080D	Epoxy-Allyl Dextran	80	Arg	62	Seph 6FF
U1689080E	Epoxy	n.a.	Arg	31	Seph Q 6FF
U1689080F	Allyl Dextran	n.a.	Arg	40	Seph Q 6FF
U1689080G	Allyl Dextran	n.a.	Arg	48	Seph Q 6FF
U1689080H	Allyl	256	Arg	91	Seph 6FF Dextran
U1689080J	Allyl	259	DEAE	205	Seph 6FF
U169221A	Mercapto propionic acid	250	Arg	45	Seph 6FF
U169221B	COOH	250	Arg	90	Seph 6FF
U169222	COOH	250	Arg	15	Seph 6FF
U169224A	COOH	250	Arg	100	Seph 6FF
U169224B	COOH	250	Arg	40	Seph 6FF
U169227A	COOH	250	ArgOEt	130	Seph 6FF
U169228	COOH	250	Agmatine	190	Seph 6FF
U169229B	NH2	147	Fmoc-Arg-OH	127	Seph 6FF
U169230B	NH2	147	Fmoc-Arg-OH	n.a.	Seph 6FF
U169231A	COOH	250	H-Arg-OEt	55	Seph 6FF
U169231B	NH2	147	Fmoc-Arg-OH	90	Seph 6FF

Table 16: Specifications of the arginine prototypes tested in microtiterplates

Seven hours after inoculation cells were only spread out on Cytodex III and started to spread on Cytodex I. Cells had attached to all of the test carriers but remained rounded. Interestingly in several cases (U1689080C, U1689080F, U1689080J, U169224B and U169230B) not even the cells attached to the plasma treated surface of the culture dish had spread out.

24 hours after inoculation the picture was essentially the same. On none of the test carriers cells had spread out. They had, however spread out on Cytodex I and on the surface of the culture dish of U16890J.

48 hours after inoculation cells began to spread on some of the test carriers (U1689080H, U169224A, U169227A, U169228, U169229B). In Figure 34 two representative pictures are shown. On U169230B the cells are still rounded after 48 h of cultivation, on U169227A some spread out cells are visible. The spreading happened most likely due to conditioning of the carrier surface by the attached but previously rounded cells. Moreover when the cells on the test carriers started to spread out the reference carriers were already confluent. As obviously none of the testcarriers showed a performance comparable to the reference carriers the experiment was terminated after 48h.

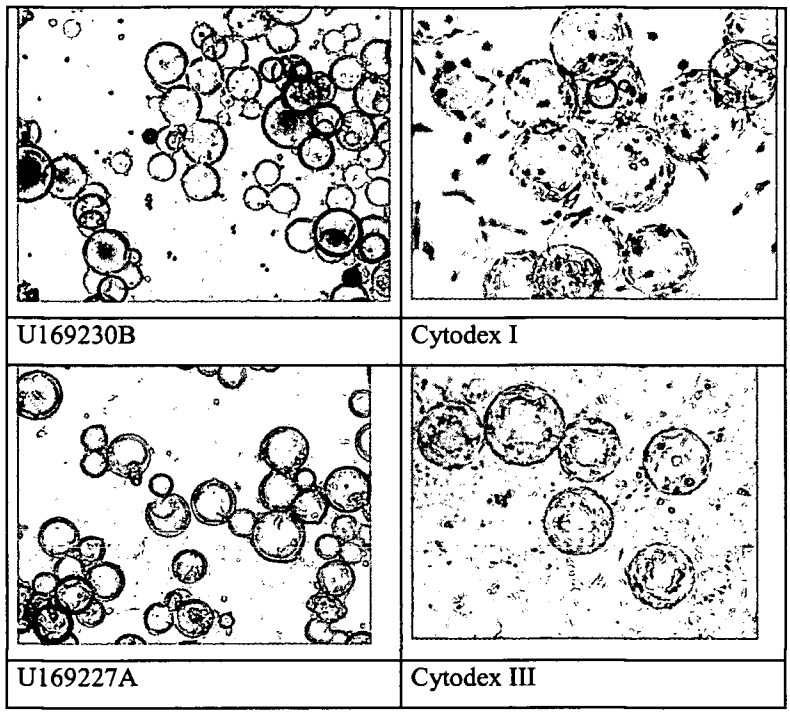


Figure 34: Cytodex prototypes after 48 h cultivation, representative pictures

In summary the testing of Cytodex prototypes in 12 well plates allowed a reduction in experiment time. Almost the fourfold number of microcarriers was tested per month compared to spinner flask experiments. In total 47 prototypes were tested. The suitability of the experimental procedure was verified by including Cytodex I and III as positive controls as well as allylated Sephadex G50 as negative control. Furthermore a parallel testing in spinner flasks and microtiterplates revealed that the results in both systems were comparable. Unfortunately the use of the GCSS was impaired by coating problems with the 96 well plates. Therefore no quantitative data on the cell growth were available. During the screening in 12 well plates 4 prototypes supported the growth on Vero cells. Two of them had DEAE at a concentration of 63 and 75 µmol/ml gel coupled to Sephadex G50. On the third successful prototype DEAE at a concentration of 115 µmol/ml gel was coupled to Sepharose 4FF. Finally also a prototype with 15 µmol/ml methyl arginine coupled to Sephadex G50 supported the growth of Vero cells.

6.1.7 Experiments with DEAE-Dextran coated Microtiterplates

In the previous experiments with immobilised carriers it had not been possible to coat the microtiterplates with a homogeneous and still transparent layer of prototypes. In the case of DEAE the ligands are not only positioned on the carrier surface but distributed throughout the matrix. This offers the possibility to coat surfaces directly with the dextran slurry instead of first producing beads that have to be immobilised afterwards furthermore no chemical treatment of the surface is needed.

In a first series 4 different dextran modifications were used to coat 12 well plates. The samples tested were: unmodified dextran and phenyl dextran as negative controls, as well as two phenyl dextrans with different amounts of DEAE coupled (U1754022A and U1754022E).

Inoculum concentration was $1.25*10^5$ cells/ml corresponding to about $7*10^4$ cells per cm^2. The cells were grown in an incubator in an atmosphere containig 7% CO2 . Cell growth was followed microscopically and images were taken 24 hours after inoculation.

The surface of the dextran treated plates remained clear and transparent with all tested dextran modifications and no significant deviation between the single wells was observed.
As shown in Figure 35 all cells were spread out in DX T40 treated wells. There was no morphological difference between the tested concentrations and cell morphology is the same as on cell culture treated plastic. The material was actually intended as negative control. It should represent an uncharged surface and no cell attachment was expected. It was verified by contact angle measurement that the dextran sticks to the plate matereial. A possible explanation for the cell growth therefore is that the applied dextran concentrations are insufficient to block interaction between cells and the surface treated polystyrene.

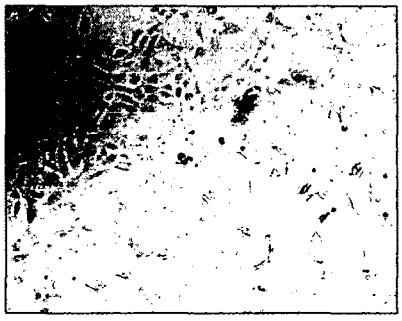


Figure 35: Cell morphology on DX T40 coated wells after 24 h

In phenyl dextran coated wells cell spreading was affected in a concentration dependent way. In wells coated with concentrations of 100, 10 and 1 mg/ml more cells showed a rounded morphology compared to a coating concentration of 0.1 mg/ml (see Figure 36).

100, 10 and 1 mg/ml	0.1 mg/ml	Vero Cells in untreated culture dish

Figure 36: Cell morphology on phenyl dextran coated plates after 24 h

Also on surfaces treated with the two DEAE-dextran modifications cell spreading depended on the concentration used for coating which reflects that a certain charge density is necessary to allow cell – surface interaction.

In U1754022A treated plates cell spreading was best at coating concentrations below 100 mg/ml. As shown in Figure 37 at 100 mg/ml cells were mostly of rounded morphology.

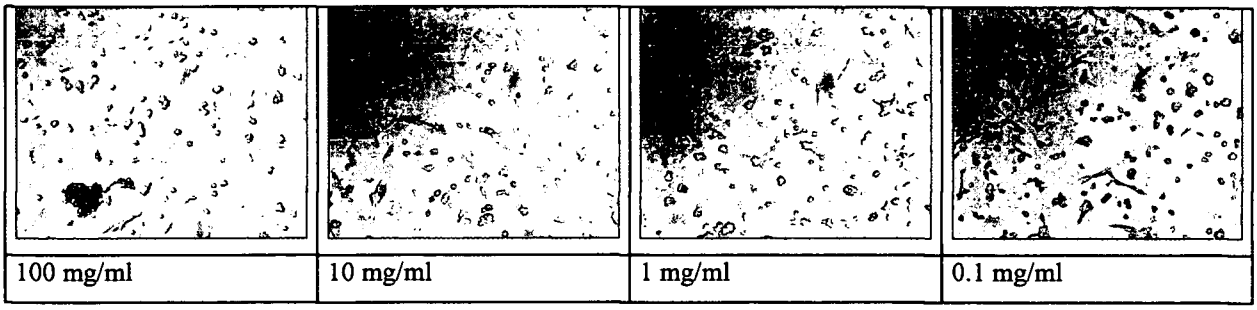


Figure 37: Cell morpology on U1754022A after 24 h

In contrast to U1754022A cell spreading was significantly better in wells treated with the two higher coating concentrations of U1754022E. In Figure 38 it can be seen that at 100 and 10 mg/ml a significantly higher percentage of cells are of flat and longitudinal shape compared to coating concentrations of 1 and 0.1 mg/ml where practically all cells are of rounded morphology.

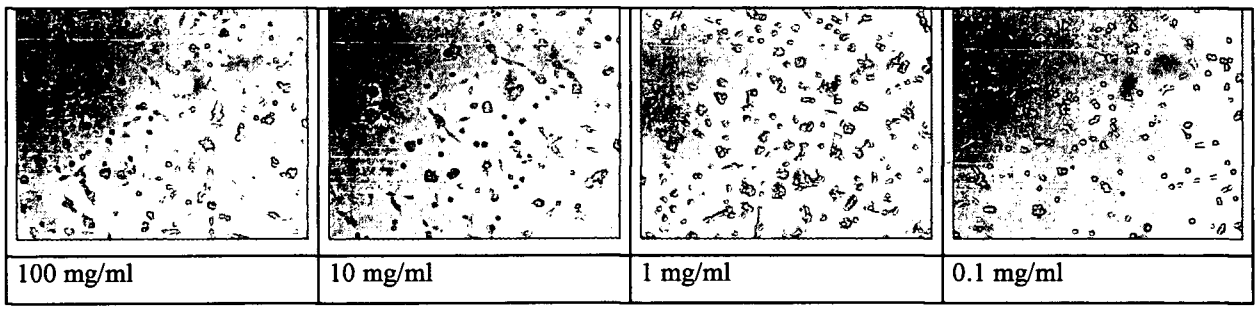


Figure 38: Cell morphology on U1754022E after 24 h

All coatings affected cell spreading in a concentration dependent manner. Phenyl dextran caused a rounded cell morphology at concentrations higher than 0.1 mg/ml, and U1754022A at a concentration of 100 mg/ml. U1754022E seems to support cell spreading as the morphology changes from rounded to long stretched at concentrations of 10 and 100 mg/ml.

6.2 Experiments on Cytopore carriers with different charge concentrations

CHO 4E10 cells were grown in spinner flasks (wv 120 ml) on 5 Cytopore carriers with different charges ranging from 0 to 1.37 meq/g. The objective of the experiment was to see the influence of the charge density on cell attachment and cell growth as well as on the specific productivity. Furthermore it should be tested if there was a toxic charge concentration for the cells. Their specifications of the prototypes are shown in Table 17. Cytopore I and Cytopore II were used as reference carriers. In total charges from 0 to 1.8 meq/g were tested.

Carrier
Cytopore 0 meq/g
Cytopore 0.26 meq/g
Cytopore 0.48 meq/g
Cytopore 0.84 meq/g
Cytopore I (1.1) meq/g reference carrier
Cytopore 1.37 meq/g
Cytopore II (1.8 meq/g) reference carrier

Table 17: Cytopore prototypes tested with CHO 4E10

Cells remaining in suspension were counted after 40 min., 80 min., 180 min. and 360 min. in order to analyse the attachment characteristics of the cells to the different carrier types. The cultures were maintained for 10 days and samples were taken daily. Starting on the second day a daily medium change of 60 ml was performed and the spinner flasks were gassed with synthetic air containing 5% CO2 for oxygen supply and pH adjustment.

As shown in Figure 39 cell counts for the Cytopore prototypes with a charge of 0 and 0.26 meq/g continually decreased for the first 5 days. When the cell number was about $1*10^5$ cells/ml no further media changes were performed. The cultures were left to stir and only counted on the 10th day of the experiment.

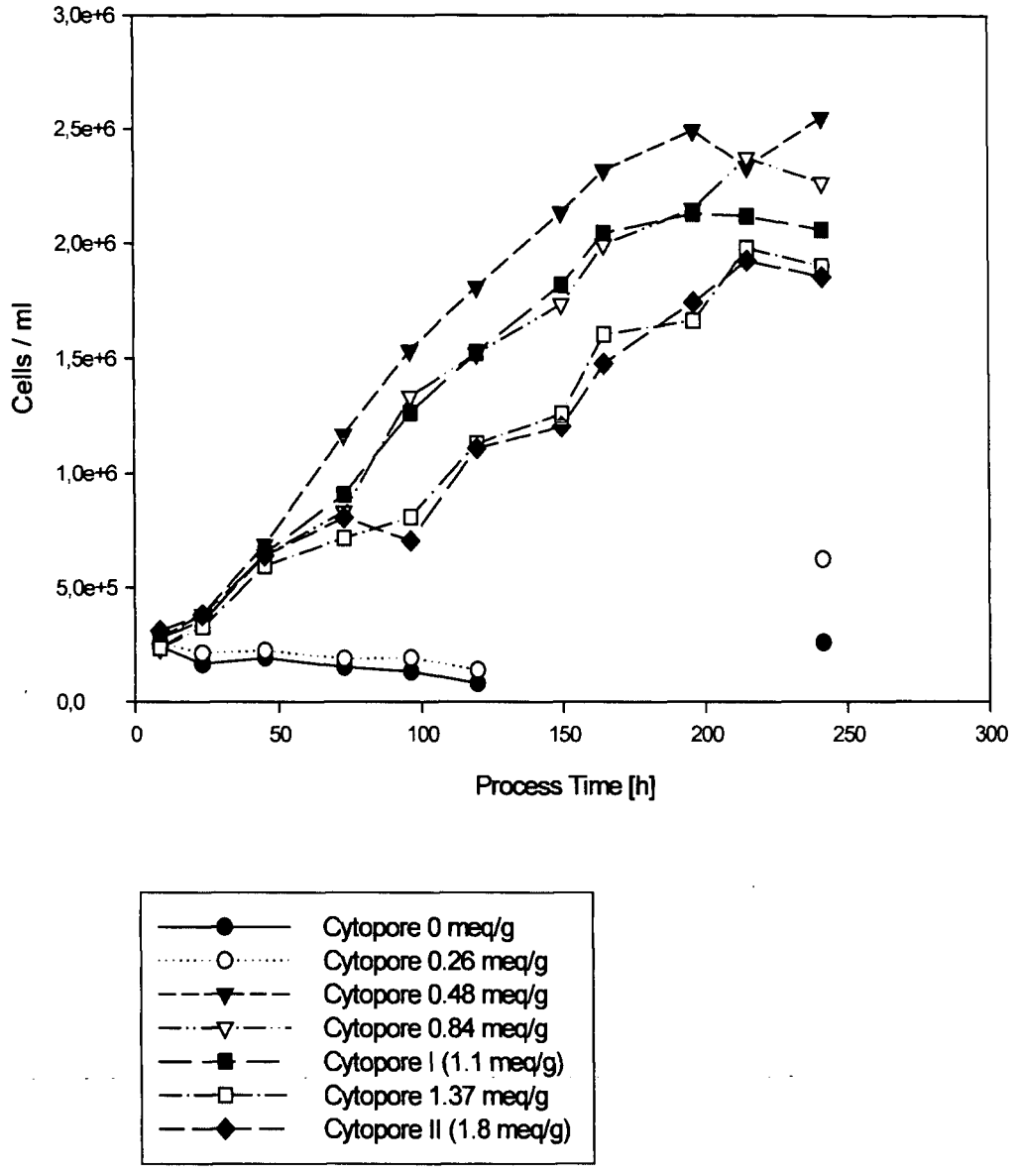


Figure 39: Cell growth of CHO 4E10 at different charge concentrations

In Figure 40 the maximum cell counts (mean value of two experiments) for the different charge densities are shown. The Cytopore carrier with 0.48 meq/g seems to be the most suitable for the cultivation of the CHO 4E10 line. At a charge density of 0.84 meq/g the maximum cell count is 7% lower and at 1.8 meq/g it drops to 76% of the maximum value. The uncharged Cytopore and the one with 0.26 meq/g do not allow sufficient interaction between cells and carriers.

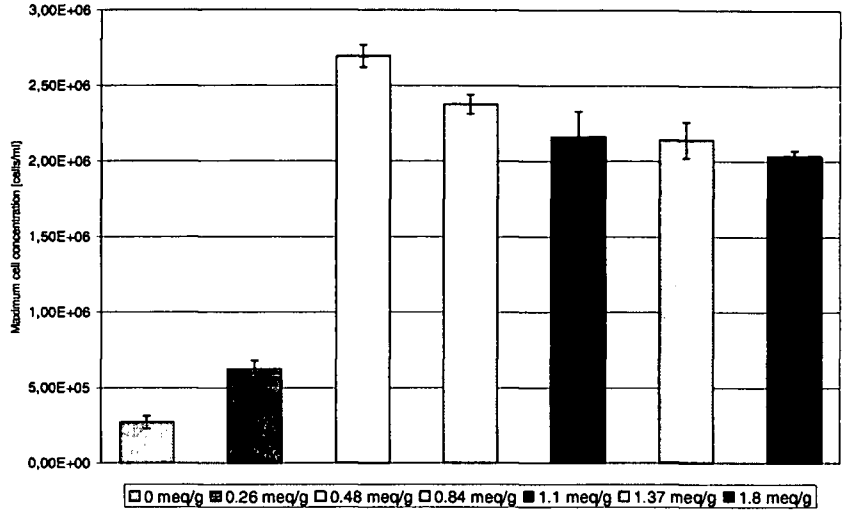


Figure 40: CHO 4E10 maximum cell counts at different charge concentrations

CHO 4E10 cells remaining in suspension (i.e. not attached to carriers) were counted after 40, 80, 180 and 360 minutes using a hemocytometer. Viability was determined by trypan blue assay. Cell count decreased quickly for carriers with a charge density of 0.48 or higher. As shown in Figure 41 at 0.84 meq/g or higher virtually all cells were attached after 80 minutes. At 0.48 meq/g it took about 180 minutes for the cells to attach completely. Cells attached poorly to the uncharged Cytopore and the one with 0.26 meq/g respectively.

Viability of the cells in suspension was higher than 90 % in all counts performed. No decrease of viability was detected over time.

Due to the reduced volume during the first 8 hours after inoculation the actual cell density was $3.65*10^5$. The first cell count of 0 meq/g was higher than this value which most probably reflects the sampling error of the method. The suspension could not be kept homogenous in order not to disturb the settled carriers.

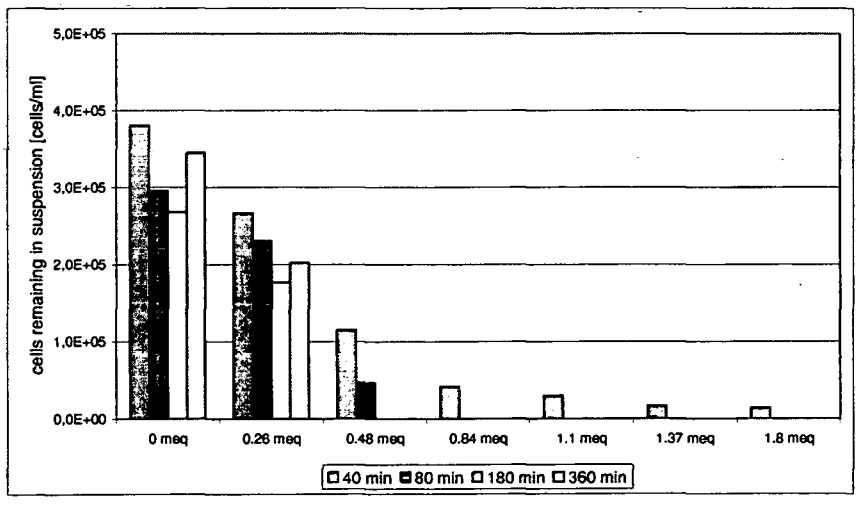


Figure 41: CHO 4E10 cells not attached to carriers at different time points

The charge dependent attachment and growth of CHO cells was verified with a second cell line. CHO 2F5 cells produce an anti HIV antibody like the previously tested CHO 4E10. The charges of the tested Cytopore modifi-

cations were slightly different to the first experiment but the same range from 0 meq/g to 1.8 meq/g was covered. The carrier specifications are shown in Table 18.

Carrier
Cytopore 0 meq/g
Cytopore 0.15 meq/g
Cytopore 0.36 meq/g
Cytopore 0.76 meq/g
Cytopore I (1,1) meq/g
Cytopore 1.37 meq/g
Cytopore II (1.8 meq/g)

Table 18: Cytopore prototypes tested with CHO 2F5

Experimental conditions and cell culture medium were the same as for the CHO 4E10 experiments. In figure Figure 42 the growth curves for CHO 2F5 on the different prototypes are shown. As can be seen in the graph charge concentrations of 0, 0.15 and 0.36 meq/g did not allow a sufficient cell-carrier interaction for attachment or cell growth. At a charge concentrations of 0.76 meq/g and above the cells grew on the carriers and high cell concentrations of around $3*10^6$ cells/ml were reached. The highest cell numbers were reached on carriers with a charge concentration of 0.76 and 1.1 meq/g . On prototypes with a charge of 1.36 and 1.8 meq/g a substantially lower maximum cell count was observed.

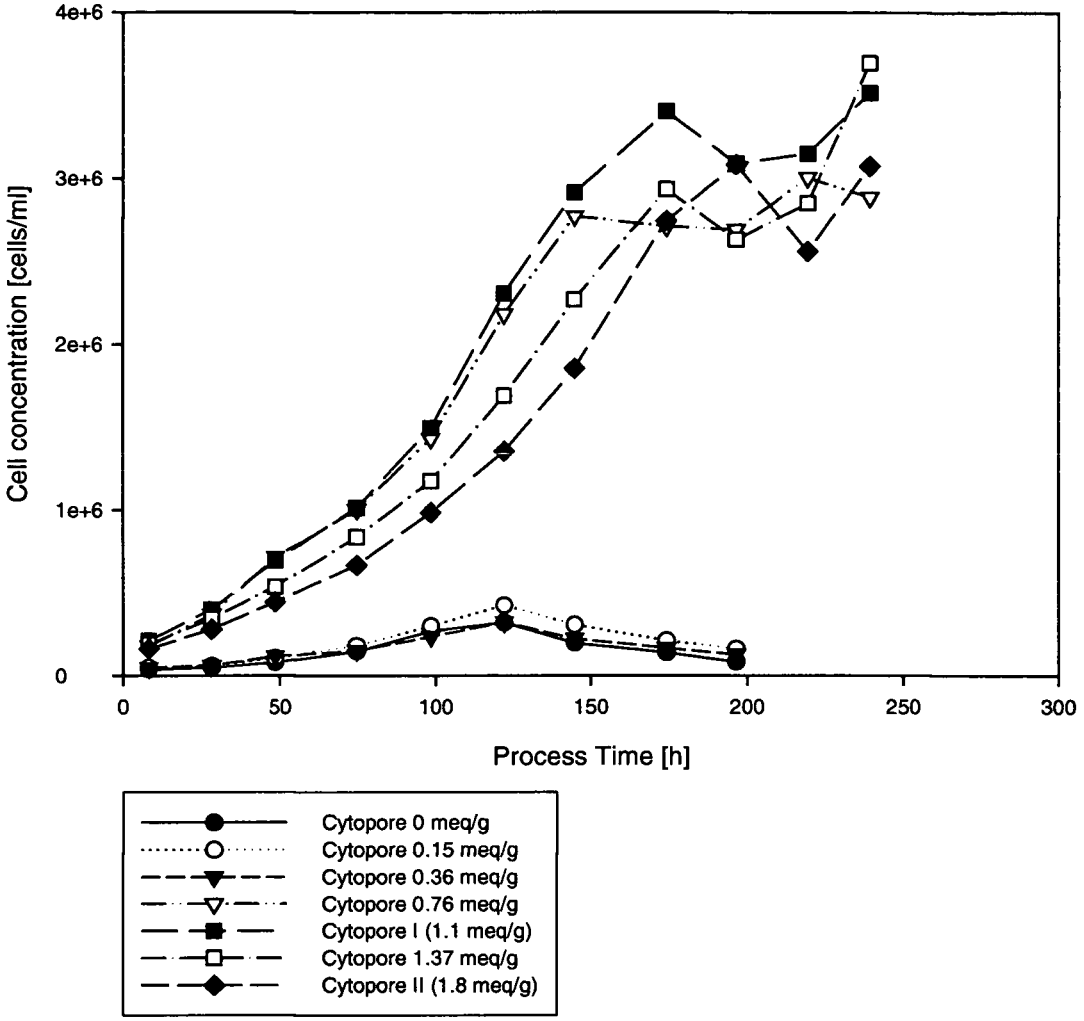


Figure 42: Cell growth of CHO 2F5 at different charge concentrations

In Figure 43 the maximum cell counts (mean value of two experiments) for the different charge densities are shown. These values were reached at 144 h cultivation time. 24 h later cells began to detach from the Cytopore carrier with 0.76 meq/g and from Cytopore I. After another 24 h cells were also found in the supernatant of the cultures with Cytopore carriers with 1.37 meq/g and of Cytopore II. Due to cell proliferation in the supernatant cell counts showed rather large variations after day 6 as depicted in Figure 42. The data were therfore not included for the determination of maximum cell concentrations.

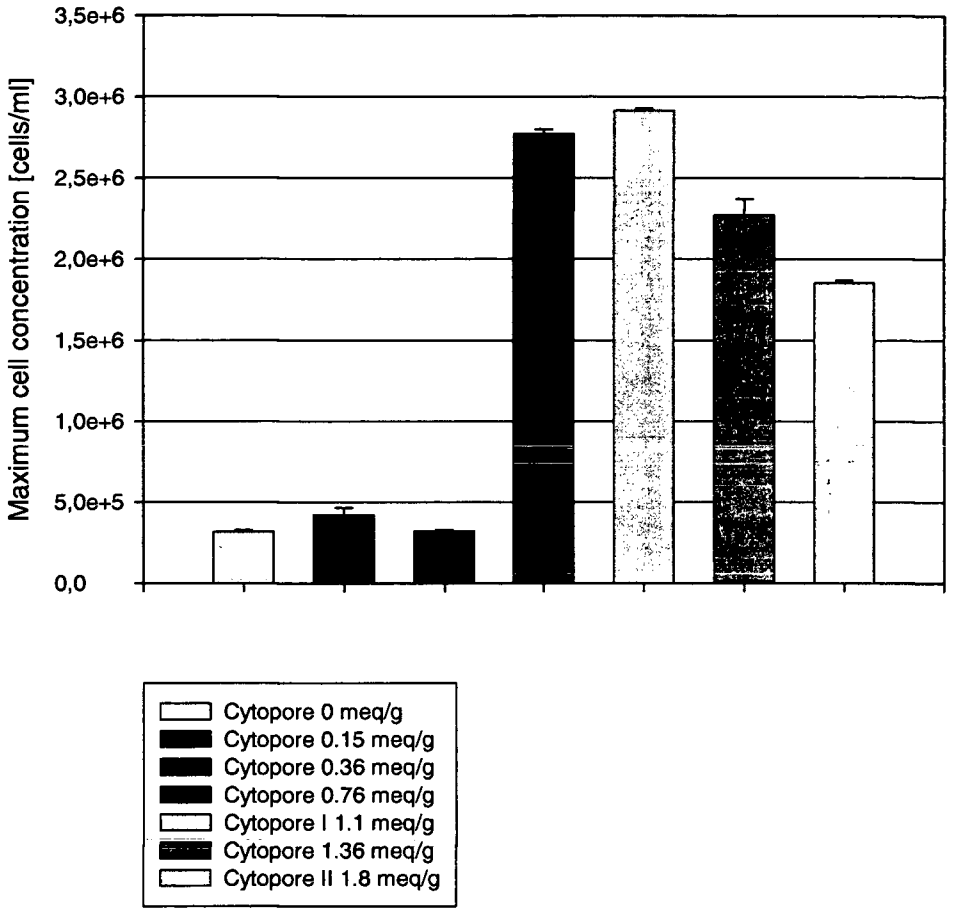


Figure 43: Maximum cell counts for CHO 2F5 on the different carrier modifications

Cells remaining in suspension (i.e. not attached to carriers) were counted after 60, 120 and 360 minutes using a hemocytometer. Viability was determined by trypan blue assay. Viability of the cells in suspension was higher than 95% in all counts performed. No decrease of viability was detected over time. In Figure 44 it is shown that CHO 2F5 cells did not attach to Cytopore carriers with a charge concentration of 0.36 meq/g or less wheras practically all cells attached to carriers with a charge concentration of 0.76 meq/g or higher within one hour.
Due to the reduced volume (62 ml instead of 120 ml) during the first 8 hours after inoculation the actual cell concentration was $4.39*10^5$ cells/ml. There were several cell counts higher than this which most probably reflects the sampling error of the method. The suspension could not be kept homogenous in order not to disturb the settled carriers.

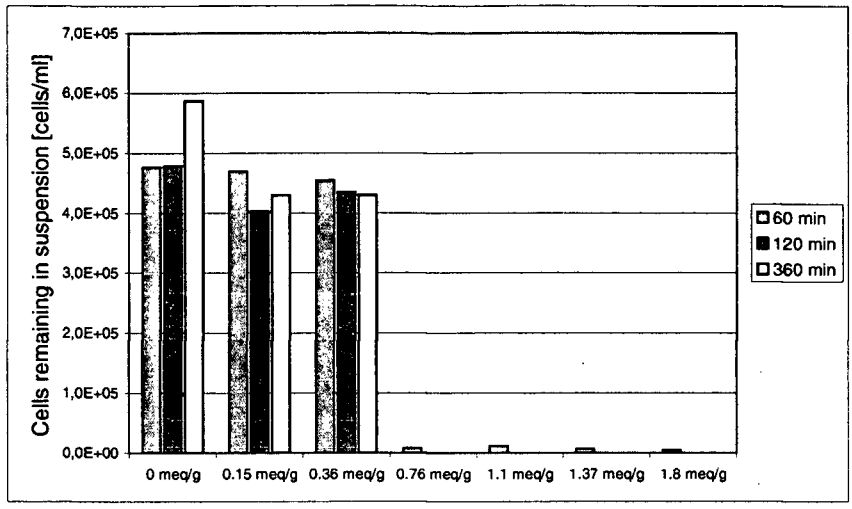


Figure 44: Cells not attached to carriers at different time points

The maximum cell number was reached on Cytopore I carriers with a charge concentration of 1.1 meq/g. However this does not necessarily mean that also the maximum antibody yield is obtained under this conditions. In order to evaluate also the productivity in the different spinner cultures antibody concentrations in the daily samples were analysed. From this data specific and volumetric productivities for the individual cultures were calculated.

As shown in Figure 45 specific productivity dropped in all experiments during cultivation time. In general after 10 days it reached about half the value of the beginning (72 h). On microcarriers with 1.37 and 1.8 meq/g this decrease was markedly slowed. Also the cell detachment that occurred during the last 72 h most probably due to the low pH began 48 h later on Cytopore prototypes with a charge concentration of 1.36 and 1.8 meq/g compared to the lower charge concentrations (data not shown). In Figure 45 the development of the specific productivity during cultivation time is shown. With 13 – 14 $pg*c^{-1}*d^{-1}$ the initial specific productivity on carriers with 1.36 and 1.8 meq/g was higher than on the carriers with 0.76 and 1.1 meq/g where the cells produced around 10 $pg*c^{-1}*d^{-1}$. Until 168 h of process time cells on the more highly charged carriers kept this elevated productivity while during the last 72 h of the experiment only cells on Cytopore II showed an increased specific productivity of 6 – 8 $pg*c^{-1}*d^{-1}$ compared to 4 – 5 $pg*c^{-1}*d^{-1}$ that were reached on the other microcarriers.

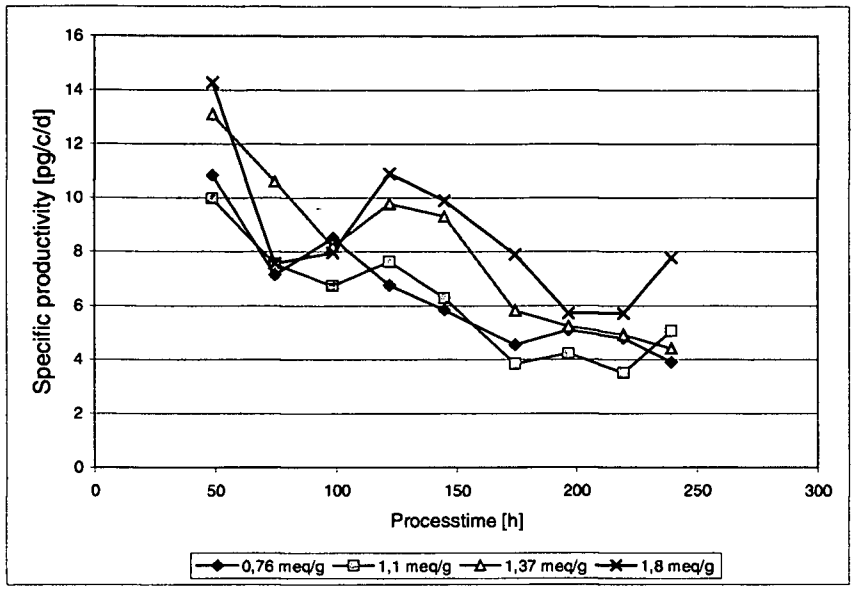


Figure 45: Specific productivity of CHO 2F5 on Cytopore modifications

The volumetric productivity of all cultures increased during the growth phase up to day 6. As shown in Figure 46 productivity on carriers with a charge concentration of 1.37 and 1.8 meq/g continued to rise for another day. The reduction occurred in parallel with the appearance of detached cells in the culture supernatant.

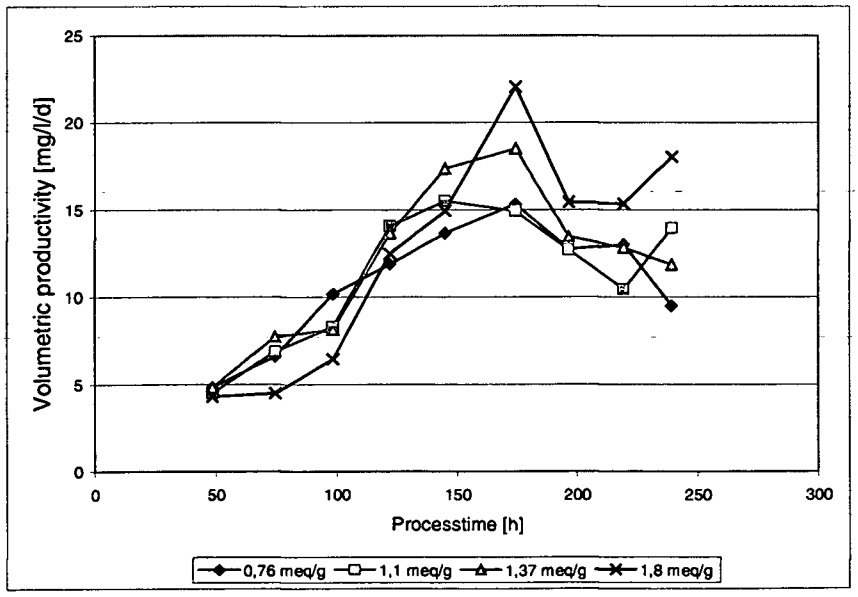


Figure 46: Volumetric productivity of CHO 2F5 on Cytopore modifications

For comparison CHO 2F5 cells from the same inoculum were also grown in suspension (see Figure 47). In Figure 48 the specific productivity of these cells is shown. No decrease in specific productivity is visible in suspension culture however the absolute values are with 6 – 8 $pg*c^{-1}*d^{-1}$ cells/d lower than the 9 – 11 $pg*c^{-1}*d^{-1}$

reached on highly charged carriers at 144 and 168 h. Especially on Cytopore II the cells show a better performance than in suspension culture until the end of the experiment.

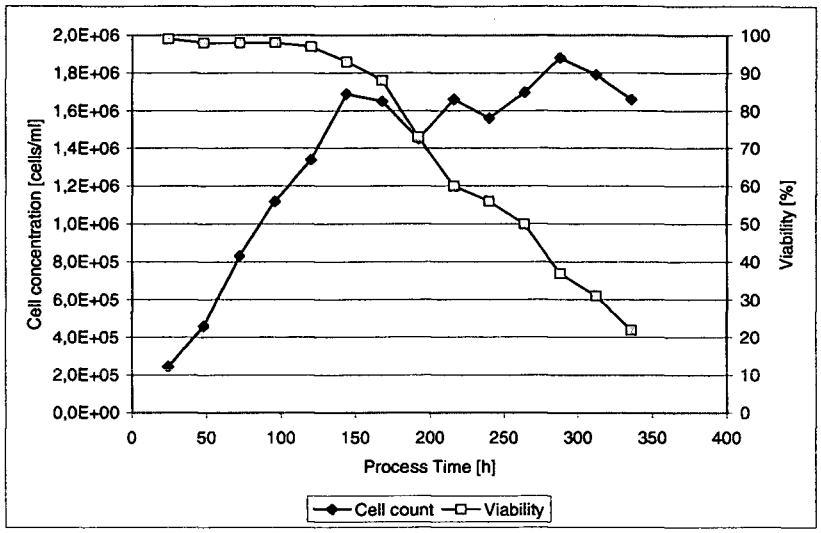


Figure 47: Batch cultivation of CHO 2F5

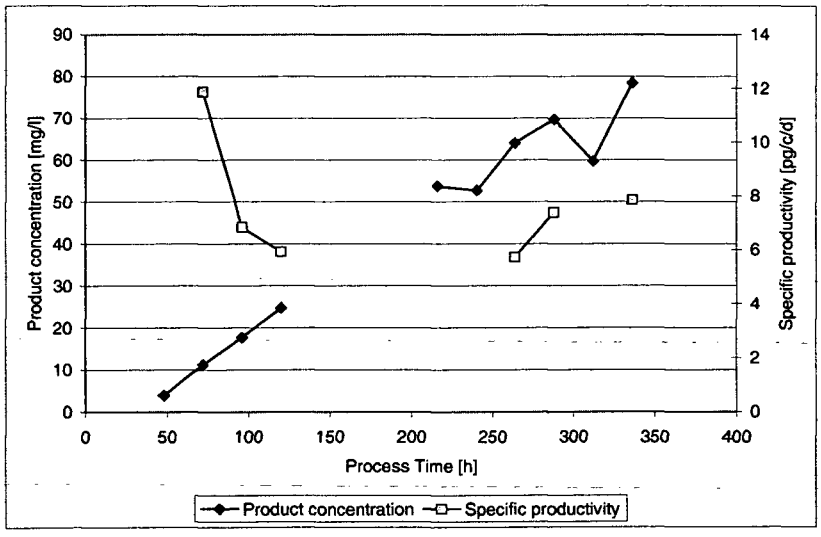


Figure 48: CHO 2F5 product concentration and specific productivity in suspension culture

The batch culture in suspension was run for 14 days, the semicontinuous microcarrier cultures for 10 days. In Figure 49 the average volumetric productivities during cultivation are depicted. The microcarrier cultures had 60 to 90% higher volumetric productivities compared to the suspension culture. The variations within the cells grown on different microcarriers were relatively small (9.77 mg/l/d to 11.36 mg/l/d) because the lower specific productivites on 0.76 and 1.1 meq/g were almost compensated by the higher cell number in these cultures. Nonetheless there is a trend visible in Figure 49 that higher charge concentrations also resulted in increased volumetric productivities. At 1.8 meq/g the average productivity was 15 % higher than at 0.76 meq/g.

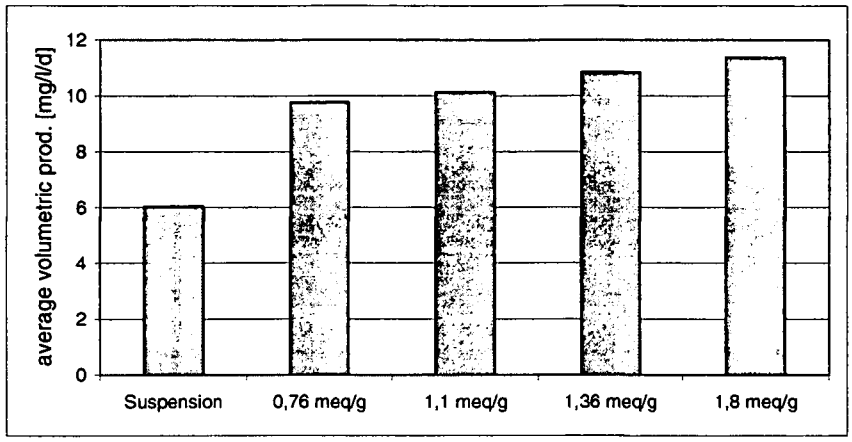


Figure 49: Average volumetric productivity during spinner flask cultivation of CHO 2F5

6.3 Bioreactor cultivations

Fermentations were conducted on Cytopore I carriers in stirred tank reactors (STR) and on Cytoline I carriers in fluidised bed reactors (FBR). For the cultivations on Cytopore carriers the carrier retention and the aeration had to be optimised. Various CHO cell lines were cultivated on both macroporous carriers. Process optimisation and media development were conducted in order to increase antibody production. The tested cell lines were CHO 2G12, CHO 4E10 and CHO 2F5 all three of them are producing an anti HIV IgG as well as CHO HB617 producing an IGM against GM1 ganglioside.

6.3.1 Cultivation of CHO 2G12

All fermentations on microcarriers were run as perfusion culture. The objective of these experiments was to test different retention devices for Cytopore carriers and compare the performance ot the cell line on Cytopore I and Cytoline I carriers. Two different devices were tried to retain the Cytopore I carriers in the reactor. The first was a sieve with a mesh size of 100 µm and a surface area of about 50 cm^2. To prevent fouling it was positioned directly above the marine impeller. An internal settler was used as second retention device. The dimensions are shown in Figure 50. The hole in the left intake socket has to be in the lee so that the streaming suspension creates a suction. The suspension is drawn in on the right intake socket. The settling zone for the carriers forms a few millimeters above the junction of the intake sockets. Due to the suction created by the streaming liquid the carriers flow over the junction and sink into the left socket where they leave the device again. The supernatant can be drawn off at the top end. With increasing flow rate the settling zone expands. During preliminary tests the device was operated at flow rates up to 12 ml/min which would allow perfusion rates of 17.2 l/d. The carriers did not accumulate inside the retention when it was run over night. However when installed in the STR the performance was not as good. Microcarriers were only retained at flow rates up to 3 ml/min. The reason for the lower retention capacity was probably that the fixtures in the reactor especially the sparger and the tubes for different filling levels increased the turbulence and prevented the forming of an uniform flow on the lee side of the device which is necessary to create a suction trough the hole in the intake socket. Consequently, at prefusion rates higher than 4.3 l/d the sieve mentioned above was used for carrier retention. It did not give any trouble at flow rates up to 12 ml/min (17 l/d) and can probably handle significantly higher flow rates but no tests were run to verify this. The loss of microcarriers through both retention systems was negligible.
Inoculum for the fermentation was prepared in spinner flasks. The working volume of the reactor was 5.5 liters.

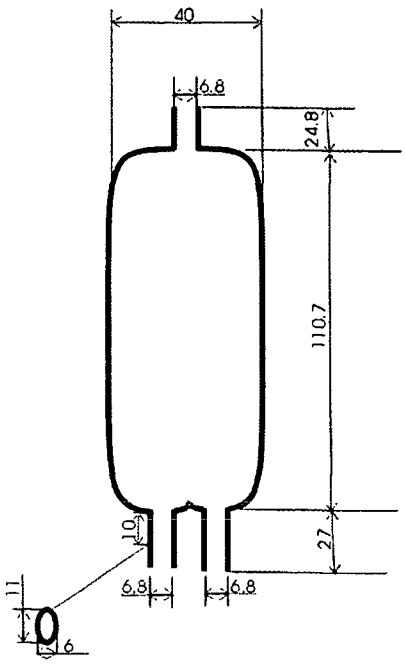


Figure 50: Settler used for Cytopore retention

For the cultivation on Cytopore I the same medium composition as for suspension cells was used except for a reduction in the ferric citrate concentration from 250 to 100 mM to facilitate cell attachment. The medium however still contained 0.1 % Pluronic F68. For aeration of the culture a microsparger with a mesh size of 0.2 µm was used.

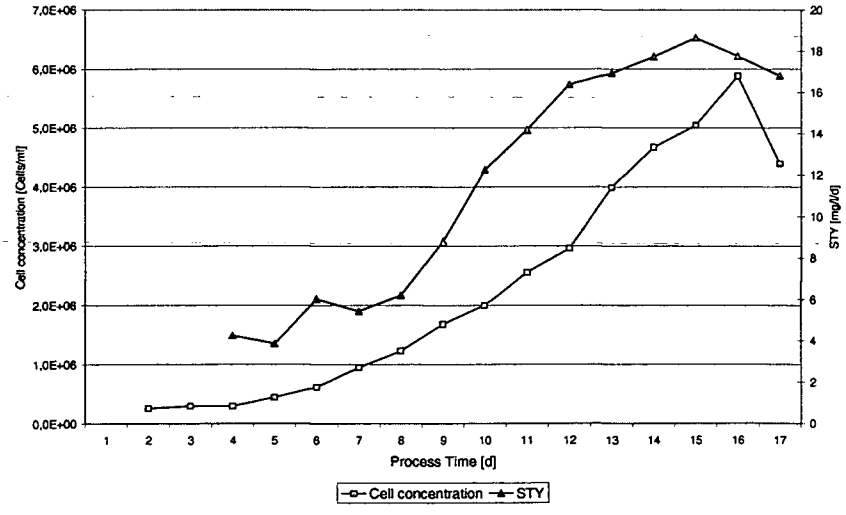


Figure 51: Cell growth and space time yield of CHO 2G12 on Cytopore I

The cells had a lag phase of about two days as can be seen in Figure 51. Cell growth started on day three. Perfusion was activated on the same day. Maybe some cell growth inhibiting component was diluted due to the perfusion. The growth rate was in the range of 0.3 – 0.4/d during the first week after that it decreased to about 0.2/d until the end of fermentation. The cell concentration increased from $2*10^5$ to $6*10^6$ cells/ml until day 15. The

drop in cell count and growth rate at the last sampling on day 16 occurred because perfusion was turned off and the residual glucose concentration dropped to 0 which resulted in cell death and detachment from the carriers.
Oxygen was originally supplied via direct sparging. However microcarriers attached to the gas bubbles and were thus at least temporarily transported into the foam layer. In accordance with cell growth also the gas flows had to be increased and the pluronic present in the culture medium stabilised the foam. On day 10 foaming was so excessive that aeration was switched from the sparger to a 6mm silicone tubing. Due to the large bubble diameter high gas flow rates had to be used. Oxygen flow started with 50 ml/min and increased up to 250 ml/min until the end of the fermentation. The high flow rates however did not seem to have a negative effect on the cell growth and foaming was considerably reduced.
As shown in Figure 52 the volumetric productivity remains at a constant low level of about 5 $mg*l^{-1}*d^{-1}$ from day 4 to day 7 because the rising cell number is compensated by a significant reduction in specific productivity. The maximum volumetric productivity of 34 $mg*l^{-1}*d^{-1}$ was reached on day 10. From that point on however cell growth did not compensate the declining specific productivity any more and so also the volumetric productivity of the system dropped to about 25 $mg*l^{-1}*d^{-1}$ for the next 3 days. The decrease to less than 10 $mg*l^{-1}*d^{-1}$ for the last two days is probably due to the reduced perfusion rate as shown in Figure 53 and the therefore deteriorating culture conditions.
Starting on day 8 the specific productivity continually decreased from 16 $pg*c^{-1}*d^{-1}$ to only 1.5 $pg*c^{-1}*d^{-1}$ on day 15. This decrease could be due to the aeration and foaming problems although cell growth did not seem to be impaired as the cell concentration increased until day 16. It is however unclear what caused the drop in specific productivity during the first 5 days. Due to the lower cell concentration the gas flow in this period was much lower and no problems with foaming occured.
As shown in Figure 53 the perfusion rate rose sharply from day 6 to day 10. On day 11 the perfusion rate was reduced from more than 3 to 2 RV/d consequently the residual glucose concentration decreased from 2000 mg/l to 1000 mg/l. The lactate concentration increased from 1500 mg/l to 2000 mg/l. The perfusion rate was then further reduced to about 0.5 RV/d on day 14 to increase the titer. This however resulted in a sharp reduction of the volumetric productivity as shown in Figure 51 and failed to increase the titer due to the drop in specific productivity. An increase in product concentration was only achieved when the perfusion was completely stopped on day 15 however this not only led to a further reduction in productivity but even to cell detachment.

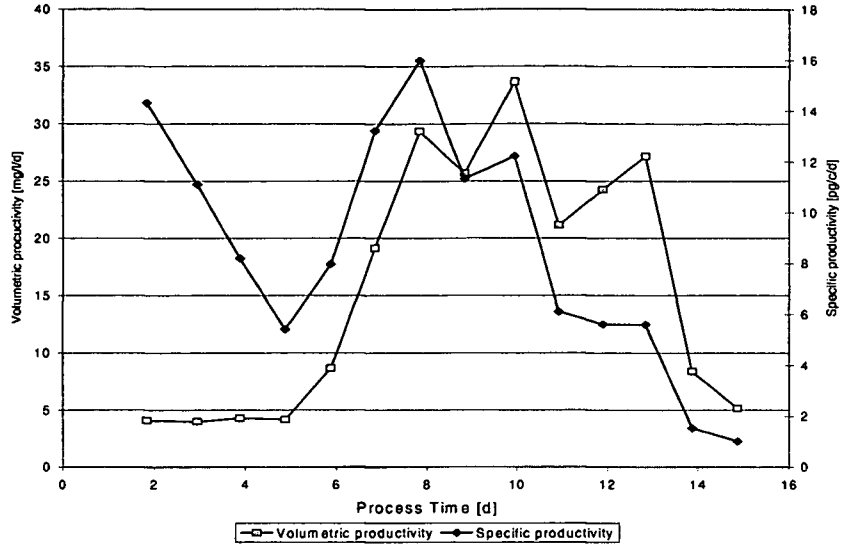


Figure 52: Specific and volumetric productivity of CHO 2G12 on Cytopore I

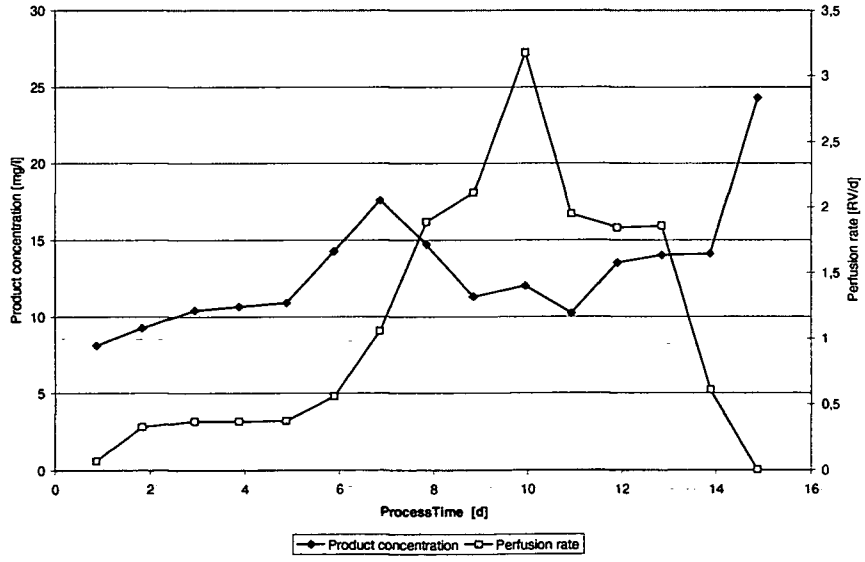


Figure 53: Product concentration and perfusion rate during cultivation of CHO 2G12 on Cytopore I

To compare the performance in a perfusion process on Cytopore and Cytoline carriers the cells were also cultivated in a fluidised bed reactor.

Cells from a continuous suspension culture were used as inoculum. Inoculum concentration was $4.8*10^6$ cells/ml carrier. After inoculation the carriers were suspended every 30 minutes for 6 hours then a 100 % media change was performed and the first carrier sample was taken. Cell attachement was only 64 % which resulted in a starting cell number of $3.07*10^6$ cells/ml carrier. The reason for the low attachment rate was probably that undiluted cell suspension from the continuous culture was used for inoculation. The growth medium for suspension cultures contained a higher concentrations of ferric citrate 250 µM compared to 100 µM in the medium used for FBR

cultivation. Furthermore Pluronic F68 was present in the medium for suspension cultivation to protect cells from shear stress. Both substances however reduce cell attachment to microcarriers.

As shown in Figure 54 cells grew and reached a maximum cell number of about $6*10^7$ cells/ml carrier on day 10. Cell growth however ceased at this point although the carriers were not fully grown. The stop coincides with the addition of a concentrate containing glucose, asparagine, cysteine, proline and soy peptone as shown in 5.1 on day 11. Glucose concentration in the medium was increased from 4.2 g/l to 6.2 g/l in order to reduce the necessary perfusion rates. At a residual glucose concentration of 1 g/l the lactate concentration increased from about 2 g/l to 3.5 g/l on day 14 and peak concentrations of 4 g/l on the days 16 and 18. This level of lactate might already be growth limiting. A negative effect of high lactate concentrations has also been seen for the CHO 2F5 cell line where cell detachment occurred at lactate concentrations higher than 3.5 g/l.

As shown in Figure 55 the specific productivity decreased from about 20 $pg*c^{-1}*d^{-1}$ on day 5 to about 2 $pg*c^{-1}*d^{-1}$ on day 13 and then stabilised at 3 – 5 $pg*c^{-1}*d^{-1}$ from day 17 to 26. The volumetric productivity however increased in parallel with the cellcount for the first 10 days. During this period cell growth obviously compensated the declining specific productivity. After the growth arrest on day 10 however also the volumetric productivity dropped from 54 $mg*l^{-1}*d^{-1}$ on day 10 to 20 $mg*l^{-1}*d^{-1}$ on day 14 and was then around 25 $mg*l^{-1}*d^{-1}$ from day 17 to 26.

In Figure 56 the development of the product concentration can be seen. It was 27 mg/l on day 4 and decreased to 9 mg/l until day 14 in accordance with higher perfusion rates and declining specific productivity. The specific productivity then stabilised and product concentration was increased by continually lowering perfusion rates as shown in Figure 56. At the end of the fermentation the perfusion rate had been lowered from a maximum of 3.5 RV/d to 0.9 RV/d. As the specific productivity remained constant the fourfold reduction of perfusion rate also led to an approximately fourfold increase in product concentration from 10 to 35 mg/ml.

Already during the first days of the culture fluidisation problems occurred. On day 4 the stirring speed was increased from 300 to approximately 400 rpm to keep the carrier bed fluidised. In the following days even more intensive agitation was needed. The stirring speed reached finally 700 rpm on day 14. On day 15 it was tried to achieve a better fluidisation by a 100 % media exchange to remove carrier debris. The stirring speed could be slightly reduced but had to be increased to 800 rpm again after 2 days. When the reactor was disassembled it was observed that a large percentage of the distributor plate's openings were clogged by carrier debris. Interestingly, the high stirring speeds did not cause a significant cell detachment. Even at 800 rpm the cell concentration in the supernatant was around $1.5*10^5$ cells/ml.

To ensure a sufficient oxygen supply despite the fluidisation problems the DO concentration was increased from 30 % to 50 % on day 4 and finally to 70 % on day 19.

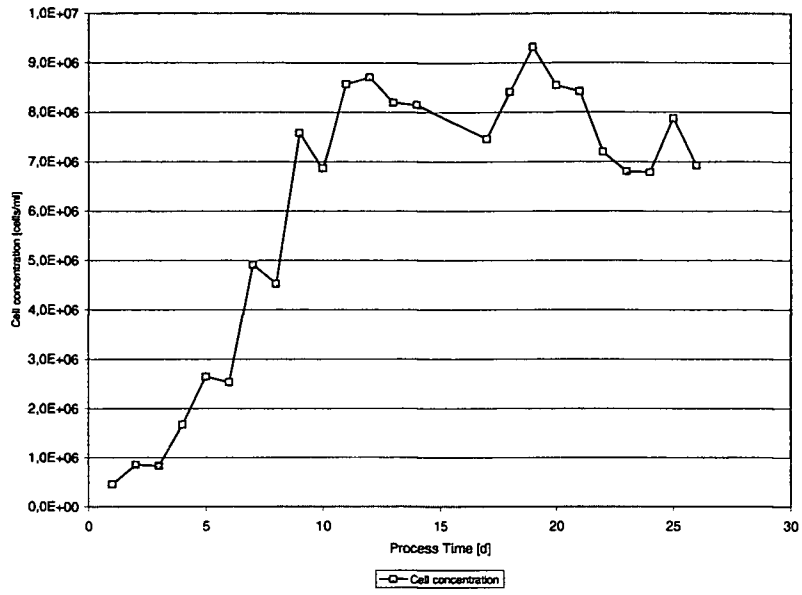


Figure 54: Cell growth of CHO 2G12 on Cytoline I

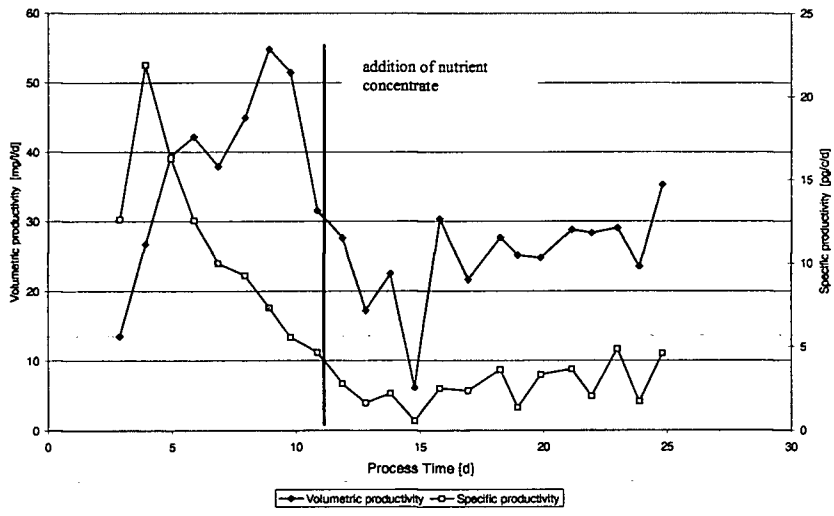


Figure 55: Specific and volumetric productivity of CHO 2G12 on Cytoline I

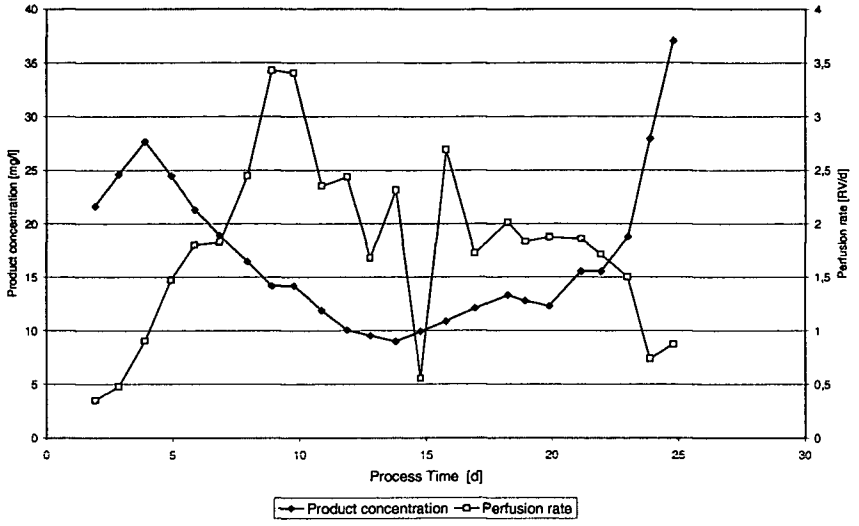


Figure 56: Product concentration and perfusion rate of CHO 2G12 on Cytoline I

6.3.2 Cultivation of CHO HB617

The objective of the cultivation of CHO HB617 cells on Cytopore I carriers was to test different retention devices. Besides the settler shown in Figure 50 also a hollow fiber cartridge run in crossflow mode was used during the perfusion phase. CHO HB617 is a CHO cell line that produeces an anti GM1 ganglioside IgM antibody. The cells are very shear sensitive which complicated the inoculum preparation. Cells were thawed and cultivated in a tissue culture flask for 3 days. Afterwards the cells were grown in a spinner flask. Besides their slow growth the cells always had a rather low viability of 80 to 90 %. When cells from spinner flasks were used for starting a bioreactor culture viability dropped quickly and no cell growth was observed. Cell viability was considerably higher (around 95 %) when no stirred systems were used for cultivation. Therefore, the inoculum for a second run in the STR was prepared in roller bottles. Cells in these cultures, however formed large aggregates. As the cells were not adapted to shear stress a higher inoculum concentration of $7*10^5$ instead of the usual $4*10^5$ cells/ml was used to shorten the conditioning of the reactor. The aggregated cells were separated by the shear forces in the STR which led to reduced viabilities of 80 to 90 % but the cells grew. They adapted to the shear stress in the course of 6 repetitive batches over 27 days. Viability during the last batch was constantly better than 93 %. Cytopore I carriers were therefore added and perfusion culture started. In Figure 57 the development of the cell counts during the course of the repeated batches and the perfusion culture is shown. The carriers were added on day 26. During suspension culture the medium contained 250 µM ferric citrate and 0.1% pluronic F68 both substances reduce cell attachment to the microcarriers therefore their concentration was halved by adding by adding medium containing no ferric citrate and no pluronic. Cytopore I were then added and 80% of the cells attached to the carriers within 2.5 h. Until day 31 the cell number increased from $3.2*10^5$ to $7.9*10^5$ cells/ml. At this point the perfusion had to be increased from 1.5 to 3.6 l/d. Due to the increased flow rate the carriers were no longer effectively retained by the gravity settler. Therefore, a hollow fiber cartridge with a surface area of 110 cm^2 and a pore size of 0.2 µm was used for carrier retention. The cartridge was autoclaved and then steamed to

an external recirculation line. Crossflow and permeate flow were both controlled by peristaltic pumps. To prevent fouling of the membrane a high crossflow rate of 532 l/d was used for a permeate flow of 3.6 l/d. The crossflow rate had been used during previous filtration experiments where crossflow filtration was used for cell separation in CHO HB617 cultures. The high flow rate however resulted in an almost 100 fold recirculation of the reactor contents per day. Due to the extreme shear stress a large proportion of the cells was killed and separated from the carriers. On day 32 the crossflow was reduced to 144 l/d and the perfusion rate was reduced to 1.4 l/d. The remaining cell number on the carriers was only $2.2*10^5$. After a lag phase of about 4 days the cells started to grow again and reached a maximum cell number of $9.8*10^5$ cells/ml on day 44. The process was ended on day 45 as the perfusion was no longer able to keep the residual glucose at a concentration of more than 1.5 g/l as can be seen in Figure 60. The perfusion volume could not be increased due to the high shear forces.

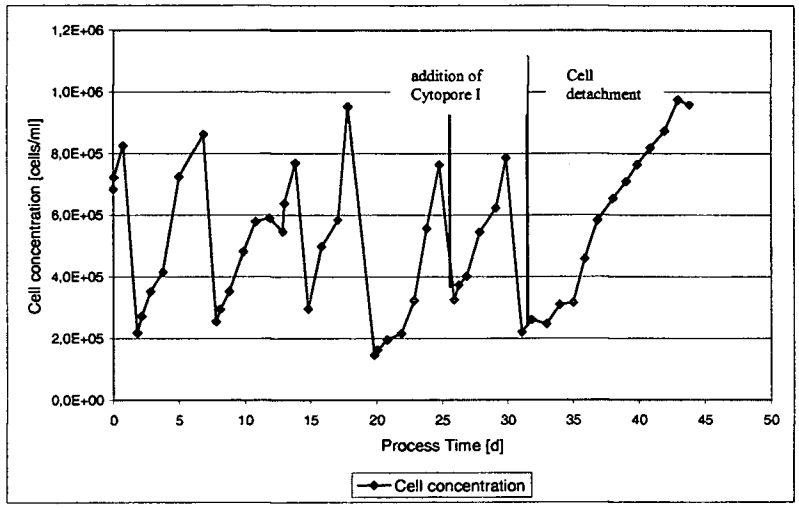


Figure 57: Cell growth of CHO HB617 on Cytopore I

In Figure 58 it can be seen that specific productivity of CHO HB617 as well as volumetric productivity increased during the cultivation. The specific productivity was significantly higher when cells were grown on carriers compared to suspension culture.

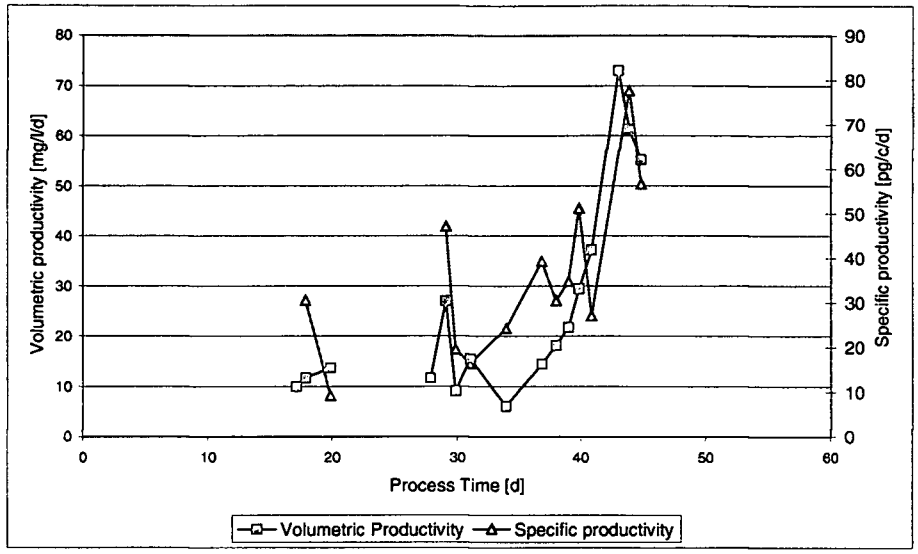


Figure 58: Volumetric and specific productivity of CHO HB617 on Cytopore I

The product concentration increased more than twofold during the cultivation on Cytopore I compared to suspension cultivation as shown in Figure 59. When volumetric productivities are compared the difference between the two cultivation modes was almost 7 fold. The perfusion culture in this case not only gave a much higher yield but also led to substantial increase in product concentration. Perfusion does not regularly lead to higher product concentrations as the medium flow causes dilution. The higher productivities therefore often have the disadvantage of low product concentrations in a large volume

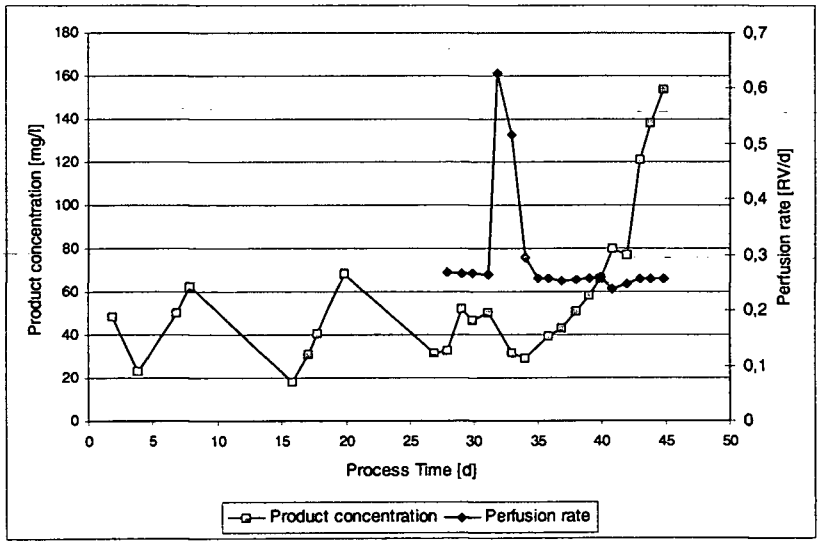


Figure 59: Product concentration and perfusion rate of CHO HB617 on Cytopore I

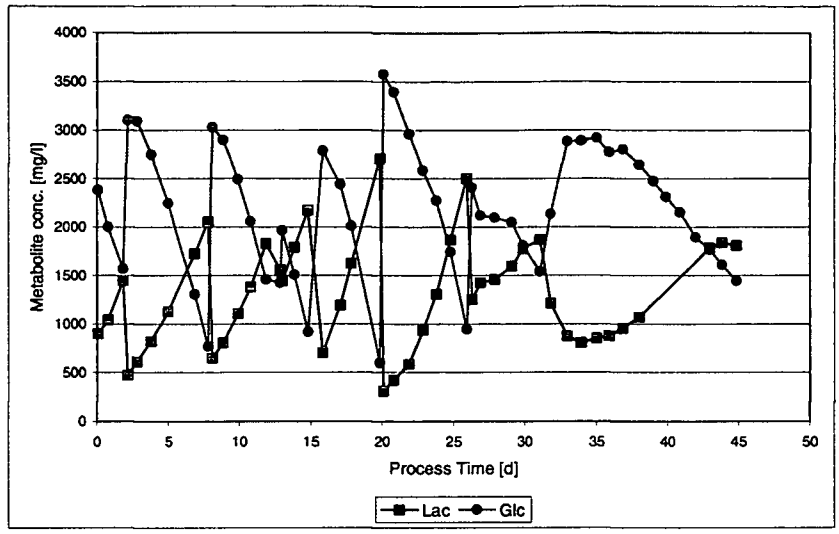


Figure 60: Development of glucose and lactate concentration during CHO HB617 cultivation

CHO HB617 cells were also grown on Cytoline I in a fluidised bed fermentation. Inoculum was taken from the suspension culture in the STR after the fourth repeated batch (see Figure 57). The viability of the cells was 90 % but there were still a lot of aggregates present. The FBR was inoculated with 1.5 l of cell suspension from the STR after 6 h 78 % of the cells had attached to the carriers. The starting concentration was $5.1*10^5$ cells/ml. The cells grew rather slowly with an average rate of 0.2/d. As shown in Figure 61 after two weeks a cell number of $6.3*10^6$ cells/ml was reached. At this point cell growth ceased although the carriers were not fully covered. In the following two weeks the cell concentration increased only marginally to about $7*10^7$ cells/ml. The reason for the low cell number might be the inhomogenous colonisation of the carriers, which occurred due to the cell aggregates in the inoculum. This led to empty areas and pores where no cell growth occurred during the cultivation. In contratst to the FBR culture of CHO 2G12 no fluidisation problems were encountered and the maximum stirring speed during 1 month of culture was 310 rpm.

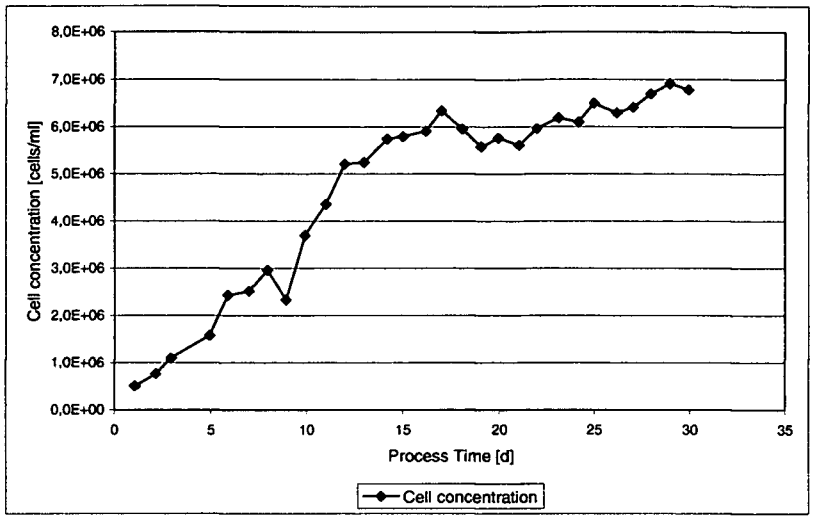


Figure 61: Cell growth of CHO HB617 on Cytoline I

Another important difference to the CHO 2G12 fermentation was that specific productivity did not drop during the cultivation but remained rather constant at 20 to 30 $pg*c^{-1}*d^{-1}$ as can be seen in Figure 62. The constant specific productivity in combination with an increasing cell number also led to a rise in the space time yield which ran practically parallel to the graph of the cell concentration. as shown in Figure 61. The same is of course also true for the volumetric productivity. Its development during cultivation is depicted in Figure 62.

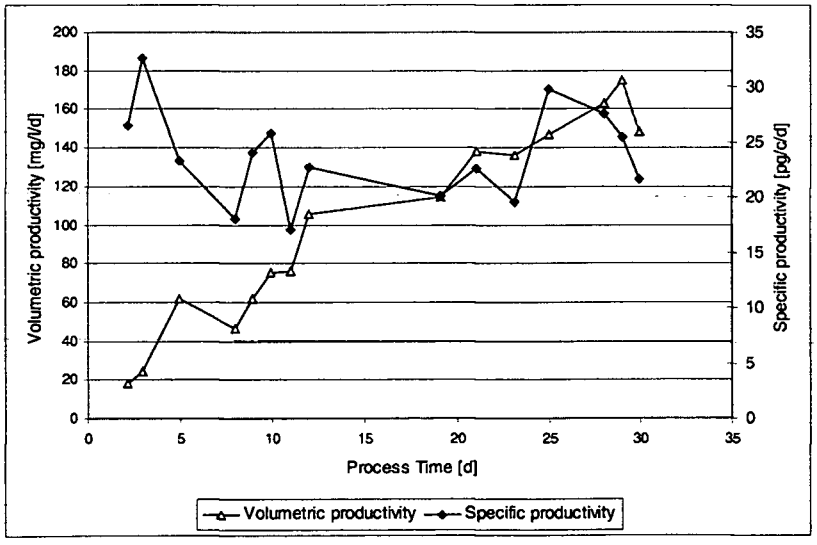


Figure 62: Specific and volumetric productivity of CHO HB617 on Cytoline 1

Perfusion was started after the cells had attached to the carriers and was then kept at half a reactor volume per day for the first 5 days. Due to cell growth and consequently increased glucose consumption residual glucose concentration dropped quickly on day 4 and 5. The Perfusion rate was increased fourfold as shown in Figure 63 so that a residual glucose concentration of 2 to 2.5 g/l was reached. Perfusion was then kept between 2 and 2.5 RV/d until day 28. As still some cell growth occured the residual glucose concentration dropped from 2.5 g/l on

day 10 to 1 g/l on day 28. During the last two days of the fermentation it was tried to reduce the glucose concentration in the harvest to 0.5 g/l in order to further increase the product concentration. As shown in Figure 63 the reduction in cell specific perfusion rate led to an about 2.5 fold increase in product concentration from day 10 until the end of the fermentation when it reached a maximum of 100 mg/l.

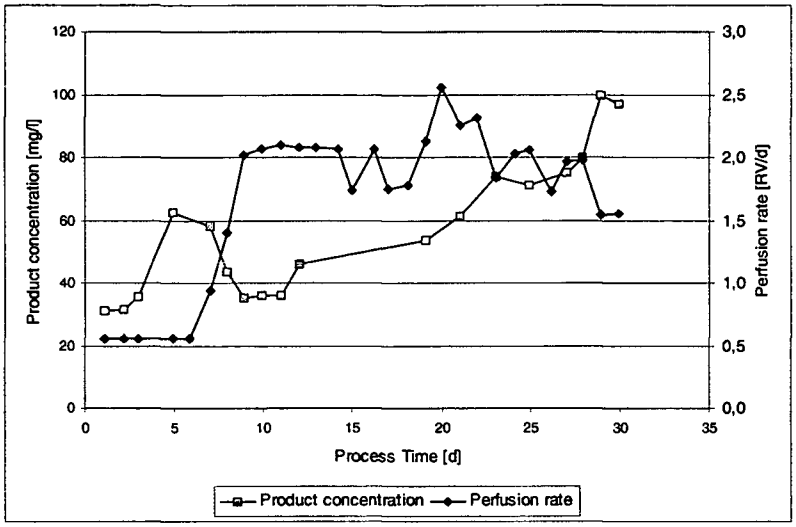


Figure 63: Product concentration and perfusion rate durintg cultivation of CHO HB617 on Cytoline I

In parallel to the declining residual glucose concentration lactate concentration increased as shown in Figure 64. Until day 20 it remained at a concentration of about 1.5 g/l. When the residual glucose concentration was lowered to 1 g/l a corresponding lactate concentration of 2.5 g/l was reached. The glucose to lactate conversion rate remained constant around 70% throughout the fermentation. Interestingly neither specific glucose consumption nor the glucose/lactate conversion rate got lower with the declining residual glucose concentration. The maximum lactate concentration was 2.7 g/l. As the specific productivity remained constant during the cultivation the increasing lactate concentration did not seem to harm the cells.

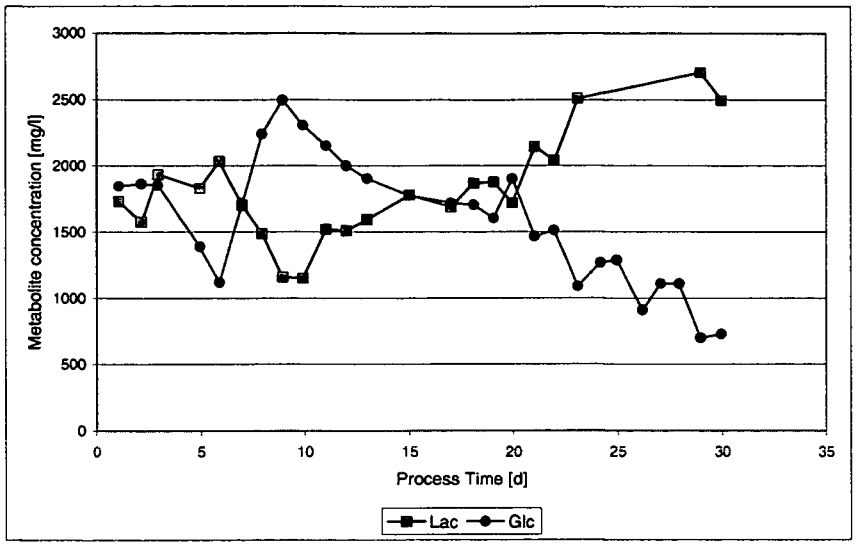


Figure 64: Development of glucose and lactate concentration during CHO HB617 cultivation on Cytoline I

6.3.3 Cultivation of CHO 4E10

The objective of the cultivation was to test the sieve retention and the performance of the hollow fiber module as carrier retention with a different cell line. It was also tried to increase the specific productivity by lowering the cultivation temperature from 37 to 34°C. CHO 4E10 is a CHO cell line producing an anti HIV IgG antibody. The cells were cultivated in a spinner flask directly after thawing. During inoculum preparation the cells had a high viability (above 94 %) but grew slowly with a growth rate of 0.3 to 0.4/d. The STR was inoculated with cells pooled from spinner flasks. Inoculum concentration was $3.7*10^5$ cells/ml. Also during cultivation in the reactor the cells showed a viability of usually higher than 95 % but grew slowly. The process was run as repeated batch until the cells had adapted to reactor cultivation. The average growth rate during the first 3 batches was around 0.3/d and the cell number at a residual glucose concentration of 1 g/l, which was the criterium for passing was only 5 to $6*10^5$ cells/ml as shown in Figure 65. The goal of adapting the cells to cultivation in the bioreactor was to reach a cell concentration of about $1*10^6$ cells/ml at the end of the batch. In the following batches cells were passed when a residual glucose of 0.5 to 1 g/l was reached. In Figure 65 it can be seen that in the batches 4 to 9 the cell concentration at the time of passing rose from $7*10^5$ to $1*10^6$ cells/ml. On day 50 two liter of cell suspension were removed for the inoculation of the FBR and Cytopore I carriers at a concentration of 2g/l were added to the STR. The reactor was perfused using the sieve retention from day 50 to day 63. At this point the sieve retention was fouled and could not be opened again by backflushing. As a hollow fiber module had been sucessfully used for carrier retention during the CHO HB617 cultivation the same solution was also tried for the CHO 4E10 fermentation. During the previous fermentation high crossflow rates had led to cell detachment it was therefore tried to use a module with larger pore size (0.45 instead of 0.2 μm) which should be more resistant to biofouling and therefore require lower crossflow rates. In the CHO 4E10 cultivations the crossflow was set to 180 l/d at a permeate flow of 7.4 l/d. Both flow rates were controlled by peristaltic pumps. After 2 days however also the hollow fiber module was blocked. On day 65 therefore half of the carriers were removed from the reactor to reduce cell concentration and therefore also perfusion requirements. For the following 18 days medium renewal was done in the following way. The necessarry amount of fresh medium for the desired residual glucose concentration was calculated. A 10 liter bottle was then aseptically connected to the reactor and the same volume of carrier suspension was transferred into it. After the carriers had settled the supernatant was discarded and the carriers were transferred back into the reactor together with the fresh medium. The CHO 4E10 cells proved to be robust and grew to a maximal cell concentration of $4.6*10^6$ cells/ml on day 71 although the carrier concentration in the reactor was only 1 g/l. In the period from day 71 to 83 the cell counts get quite unsteady probably because cell growth slows down and fluctuations in the cell number caused by removing carriers during medium renewal are no longer compensated. At the end of the fermentation the volume of the settled carriers was measured to be 120 ml which corresponds to a carrier concentration of 0.5 g/l. Thus half of the carriers have been lost during the 18 days of manual media change.

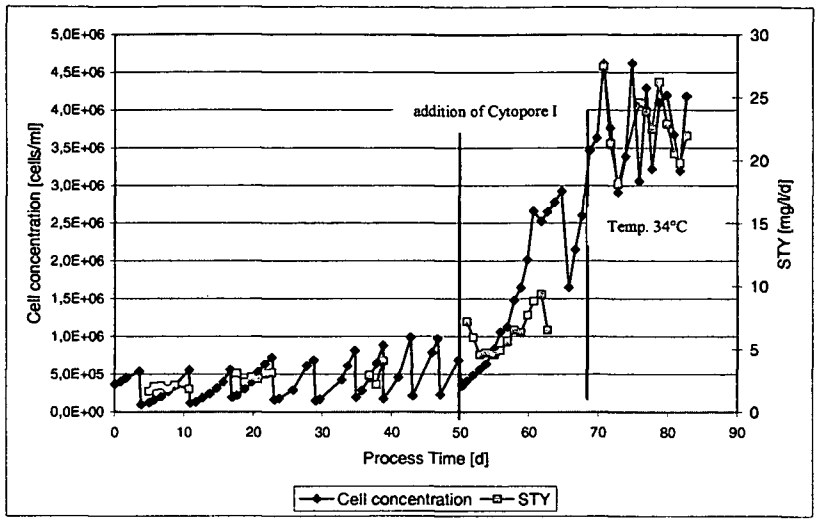


Figure 65: Cell growth and space time yield of CHO 4E10 in suspension culture and on Cytopore I

During suspension culture the maximum volumetric productivity was about 5 $mg*l^{-1}*d^{-1}$ as shown in Figure 66 the higher value of 8 $mg*l^{-1}*d^{-1}$ on day 39 is an outlier caused by the low ELISA result on day 38. During perfusion cultivation the volumetric productivity rose parallel to the cell number from day 50 to 62. During the first 10 days of perfusion culture a fourfold increase to about 20 mg/l/d on day 60 was achieved. The maximum value of 25 $mg*l^{-1}*d^{-1}$ was reached on the days 71 and 76.

On day 69 the temperature in the STR was lowered from 37 to 34°C to reduce glucose consumption and hence the necessarry perfusion volume. The cell concentration from day 71 to 83 was about 50 % higher than from day 61 to 65 the volumetric productivity however did not increase to the same extent. This was due to the reduction of the specific productivity which declined by some 20% if the periods from day 50 to day 60 and from day 70 to 83 are compared. As shown in Figure 66 the specific productivities fluctuate quite heavily between about 6 and 10 $pg*c^{-1}*d^{-1}$ due to some inconsistent ELISA results that could not be clarified by repetition of the analysis.

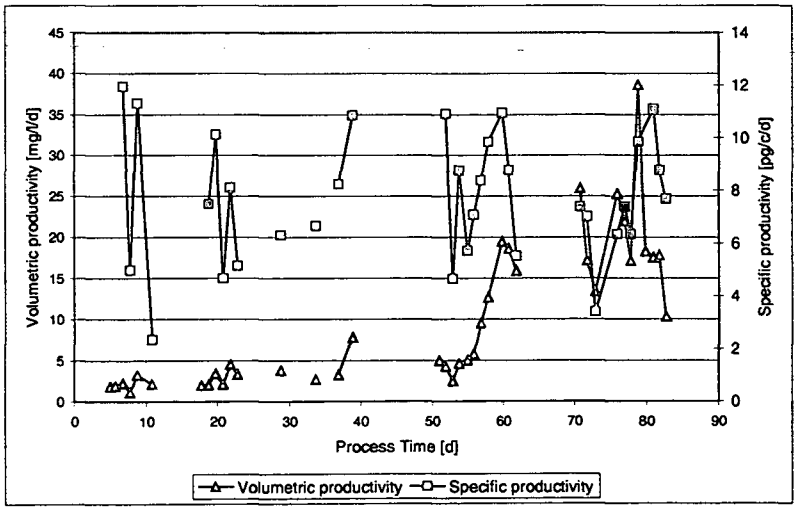


Figure 66: Specific and volumetric productivity of CHO 4E10 in suspension culture and on Cytopore I

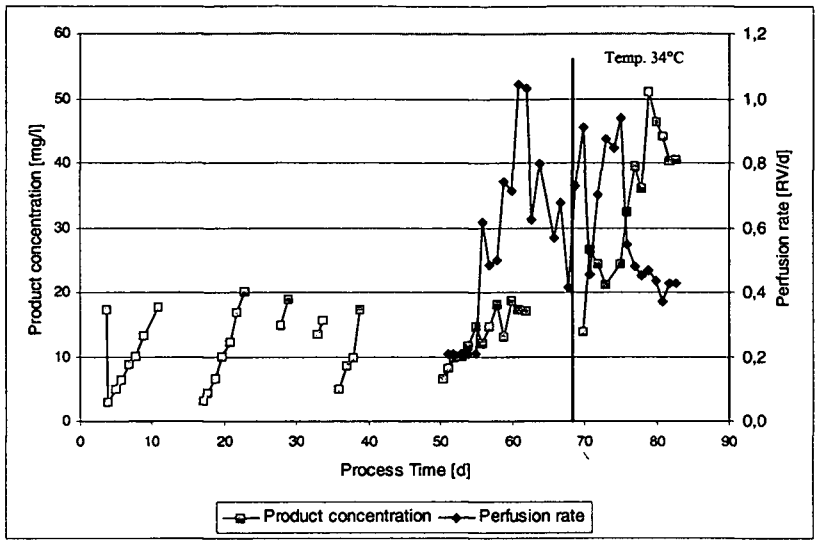


Figure 67: Product concentration and perfusion rate during CHO 4E10 cultivation in suspension and on Cytopore I

During suspension culture the product concentration at the end of the repetitive batches was around 20 mg/l. It remained the same concentration during the first phase of the perfusion culture until day 62 as shown in Figure 67. Only after perfusion was significantly reduced on day 76 the product concentration increased to about 40 to 50 mg/l. However the volumetric productivity remained rather constant at values from 20 to 25 $mg*l^{-1}*d^{-1}$ Until day 76 the residual glucose concentration was kept at about 1.5 g/l. As shown in Figure 68 the corresponding lactate concentration was about 2g/l. Glucose concentration in the medium was 4.2 g/l. The glucose to lactate conversion rate was about 70% throughout the perfusion culture. It did not change with the lowering of the residual glucose concentration to about 0.5 g/l after day 77 which was done to reduce perfusion rate and increase product concentration accordingly.

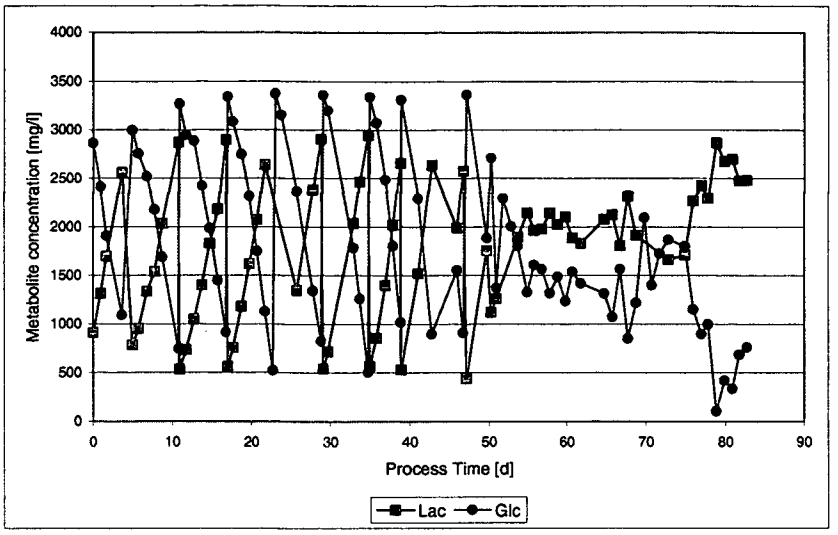


Figure 68: Development of glucose and lactate concentration during CHO 4E10 cultivation in suspension and on Cytopore I

To compare the performance of CHO 4E10 cells on different carriers they were also cultivated on Cytoline I in a FBR. The culture was inoculated with 2 liters cell suspension taken from the aforementioned STR the cells were therefore exactly in the same condition when they were added to the Cytoline and Cytopore carriers respectively. After 6 h about 70 % of the cells had attached to the Cytoline 1 carriers. The starting cell number was $3.8*10^5$ cells/ml. Cell growth was steady with an average growth rate of 0.26/d until day 14 at this time a cell number of $1*10^7$ cells/ml was reached. As shown in Figure 69 from then on no significant cell growth occurred until the end of the fermentation although the carriers were not fully grown. The sudden onset of this growth arrest suggests some problem in the culture conditions however no obvious reasons were noted. The residual glucose concentration was around 1.5 g/l with a corresponding lactate concentration of about 2 g/l and there were no fluidisation problems. As cell growth had ceased the temperature was lowered from 37 to 34°C on day 19 in order to reduce the necessarry perfusion rate and thus increase product concentration. During the following 4 days the antibody concentration increased as intended but the fermentation had to be terminated due to failure of the stirring motor on day 24.

In Figure 69 it can be seen that the total productivity rose practically parallel to the increasing cell number. Temperature reduction to 34°C led to a significant increase in specific productivity from 2.5 to about 3.5 $pg*c^{-1}*d^{-1}$ as shown in Figure 70. As the cell number remained constant this is also reflected in a rise of the volulmetric productivity from 30 to 45 $mg*l^{-1}*d^{-1}$ between day 19 and 23.

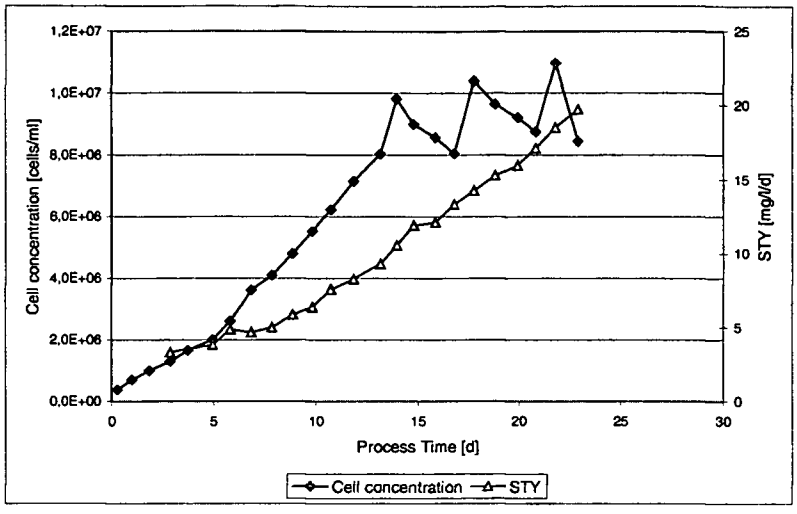


Figure 69: Cell growth and space time yield during cultivation of CHO 4E10 on Cytoline I

Nonetheless the maximum specific productivity on Cytoline I was less than half of the one achieved on Cytopore I carriers. Interestingly, the temperature reduction produced the opposite effect in the two cultivation systems. While there was a significant increase in specific production in the FBR cells in the STR even reduced their productivity slightly.

In Figure 69 the development of the space time yield during the cultivation is shown. As the specific productivity remained around 2.5 $pg*c^{-1}*d^{-1}$ it increased in parallel with the cell concentration. The rise of the specific productivity from 2.5 to 3.5 $pg*c^{-1}*d^{-1}$ is not expressed in this parameter as it reflects the average productivity between a definite point during cultivation and the start of the culture.

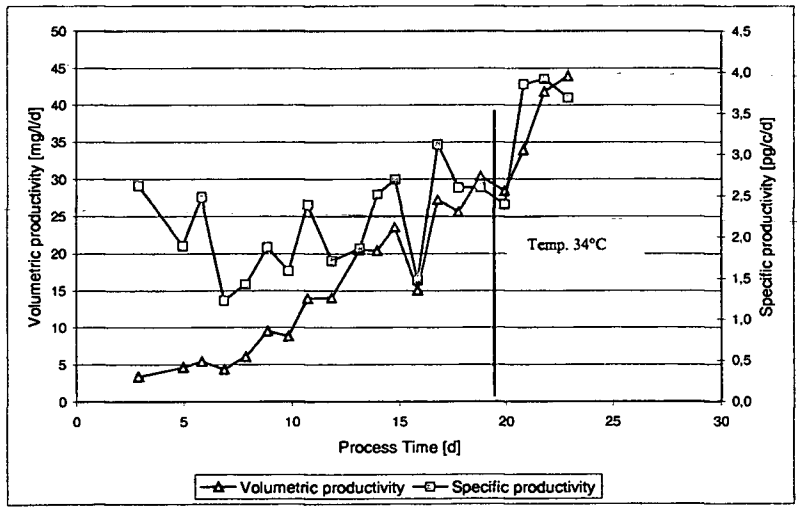


Figure 70: Specific and volumetric productivity of CHO 4E10 on Cytoline I

In Figure 71 it can be seen that the perfusion rate increased sharply from 1 to 8 l/d in the period from day 5 to 16. After the temperature reduction on day 19 it was lowered to about 6.5 l/d. As shown in Figure 72 the residual glucose concentration remained at about 1.5 g/l throughout the fermentation.

Product concentration was very low at about 6 mg/l during the first two weeks of the fermentation. As shown in Figure 71 it was doubled to 12 mg/l by reducing the perfusion rate between day 19 and 23. The maximum product concentration was less than 25 % of the one achieved on Cytopore I and did not even reach the 20 mg/l that were obtained in the repetitive batches during suspension culture.

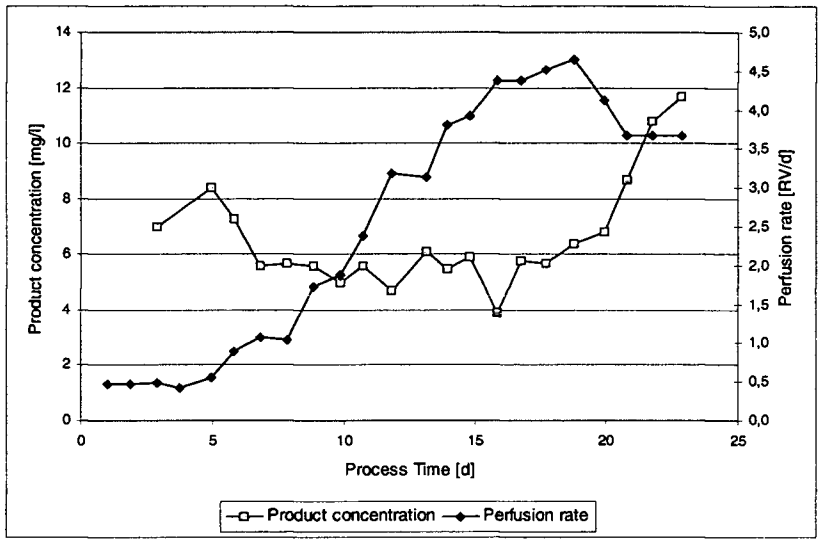


Figure 71: Product concentration and perfusion during CHO 4E10 cultivation on Cytoline I

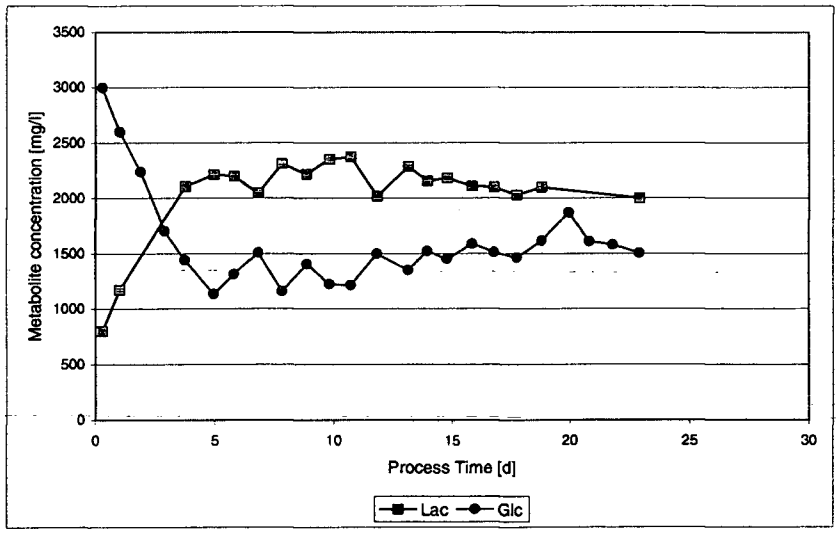


Figure 72: Development of glucose and lactate concentration during CHO 4E10 cultivation on Cytoline I

6.3.4 Cultivation of CHO 2F5

The objective of this cultivation series was to increase the product concentrations in the perfusion systems. During the preceeding cultivations the product concentrations were very low with the exception of the CHO HB617 cell line, which reached an antibody concentration of 100 mg/l. In order to increase the total productivity experiments were performed to increase the specific productivity by reduction of the cultivation temperature or by addition of sodium butyrate. To increase the product concentration an optimisation of the cultivation medium was done to reduce the necessary perfusion rates.

The CHO cell line 2F5 produces an anti HIV IgG antibody. The cells were grown in a tissue culture flask after thawing and transferred to a spinner flask after 2 passages. The spinner flask was run as backup in parallel to the cultivation in the bioreactor. During the first 60 days after thawing the medium growth rate in the spinner flask was 0.7/d and the specific productivity about 7 to 9 $pg*c^{-1}*d^{-1}$. Between day 60 and 70 the specific productivity declined from 7 to 5 $pg*c^{-1}*d^{-1}$ and finally to 2 – 3 $pg*c^{-1}*d^{-1}$ on day 90. No apparent reason for the decrease in specific productivity was found.

On day 7 and 90 of the cultivation in the backup spinner cells were removed for batch cultivation in spinner flasks. As shown in Figure 73 the maximum cell count during the first batch was $1.8*10^6$ cells/ml (maximum viable cell count $1.57*10^6$ cells/ml) in the second batch the maximum cell concentration was almost doubled to $3.0E*10^6$ cells/ml (maximum viable cell concentration $2.56*10^6$ cells/ml). The cumulated viable cell days consequently increased 2.4 fold from $1.17*10^7$ to $2.83*10^7$ $cell*d*ml^{-1}$. In Figure 74 the development of growth rate and specific productivity is shown. While no significant differences in the growth rates are visible the specific productivity was reduced from 5 – 7 $pg*c^{-1}*d^{-1}$ to only 1.5 – 2 $pg*c^{-1}*d^{-1}$ in the second batch. This resulted in a 60% reduction of the maximum product concentration from 70 to 24 mg/l as shown inFigure 73. The specific consumption rates especially of glucose and glutamine did not show significant differences in the two cultivations. However in the second batch the cells continued to grow after glutamine was consumed on day 5 and the cell number still increased from $2.2*10^6$ cells/ml to $3.0*10^6$ cells/ml wheras during the first batch cell growth stopped after glutamine was spent and the cell concentration remained at $1.7*10^6$.

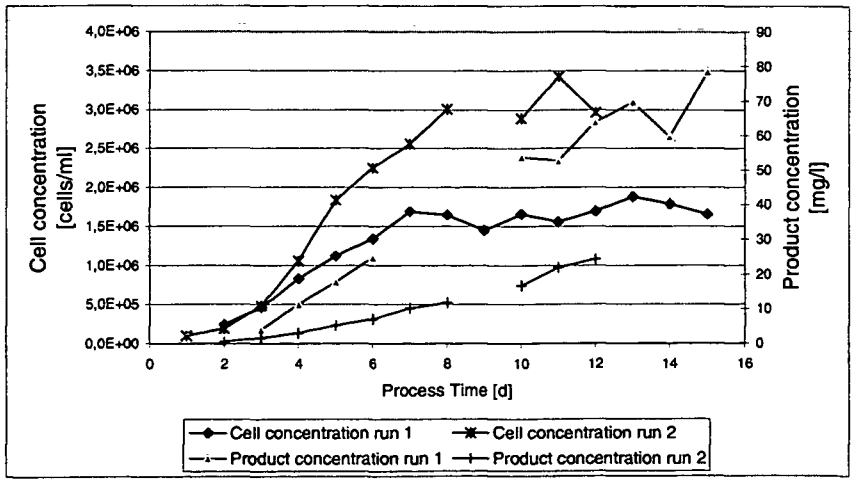


Figure 73: CHO 2F5 batch cultivation inoculated from backup spinner after 7 and 90 days of cultivation

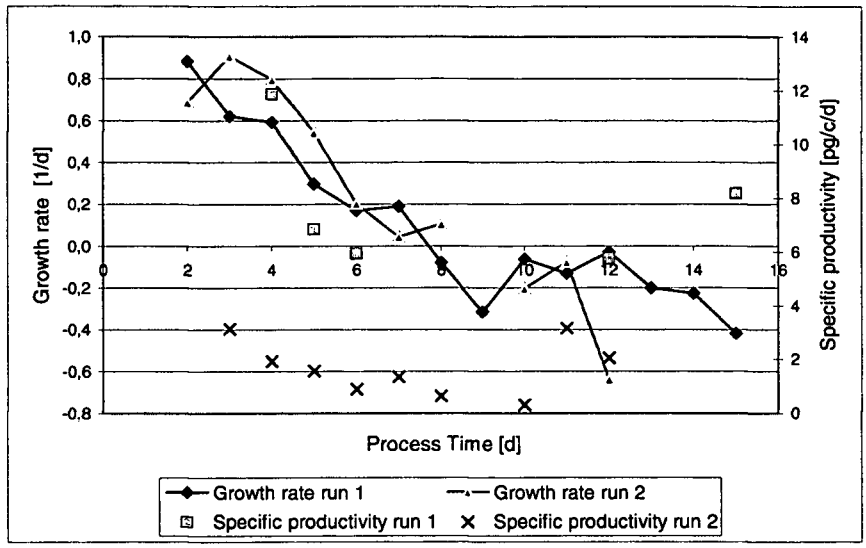


Figure 74: growth rate and specific productivity during CHO 2F5 batch cultivation

The objective of the CHO 2F5 cultivation was to test the performance of the cell line on Cytoline I and Cytopore I carriers and to see the impact of a reduced cultivation temperature on the specific productivity. Furthermore also the composition of the cultivation medium was optimised. The cells were first cultivated in a FBR. Inoculum was grown in spinner flasks and the cells were transferred into the reactor on day 14 of the backup culture. The FBR was inoculated with 1.3 l cell suspension, 60 % of the cells had attached to the Cytoline I carriers after 6 hours and the cell concentration was $4.6*10^5$ cells/ml. During the first two weeks the cells grew with a medium rate of 0.3/d and had reached a cell concentration of $2*10^7$ cells/ml on day 14. As shown in Figure 75 the cell count remained in this range for a further week until day 22. In an effort to reduce perufsion rate and thus increase product concentration the cultivation temperature was lowered from 37 to 33°C on day 23. The cell count dropped slightly below $1.5*10^7$ cells/ml carrier during the next 3 days but then stabilises around 1.5 to $1.7*10^7$ cells/ml until day 40. On day 31 the glucose concentration in the medium was increased from 3.1 g/l to 4.7 g/l by adding a concentrate containing glucose, soy peptone and the amino acids glutamate, aspartate, cysteine and asparagine (see 5.1 for the exact formulation). The cell count then remained around 1.0E+08 cells/ml carrier until day 43. As the expected increase in product concentration had not been achieved the temperature was again risen to 37°C and the glucose concentration was lowered to 4 g/l. This however resulted in cell detachment and the cell concentration dropped to about $7*10^6$ cells/ml. Also in the following days the culture did not recover and cell number remained below $1*10^7$ cells/ml. Due to the dissatisfying product concentration the culture was then terminated after 50 days.

In Figure 75 it is clearly shown that the total productivity during the first 3 weeks strictly follows the increase in cell number. However after the temperature reduction on day 23 it is halved from about 120 to 60 mg/d. After the increase in glucose concentration on day 31 it is further reduced to about 20 mg/d. Finally after readjustment of the temperature to 37°C on day 43 there is practically no antibody produced any more.

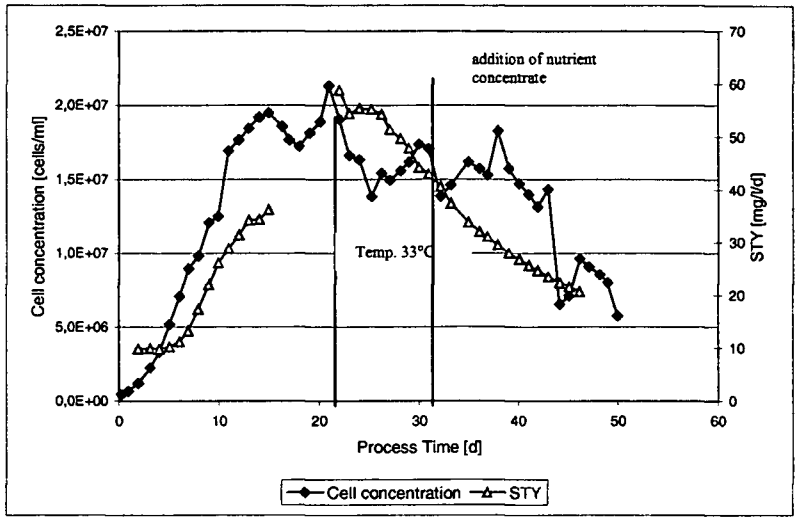


Figure 75: Cell growth and productivity of CHO 2F5 on Cytoline I

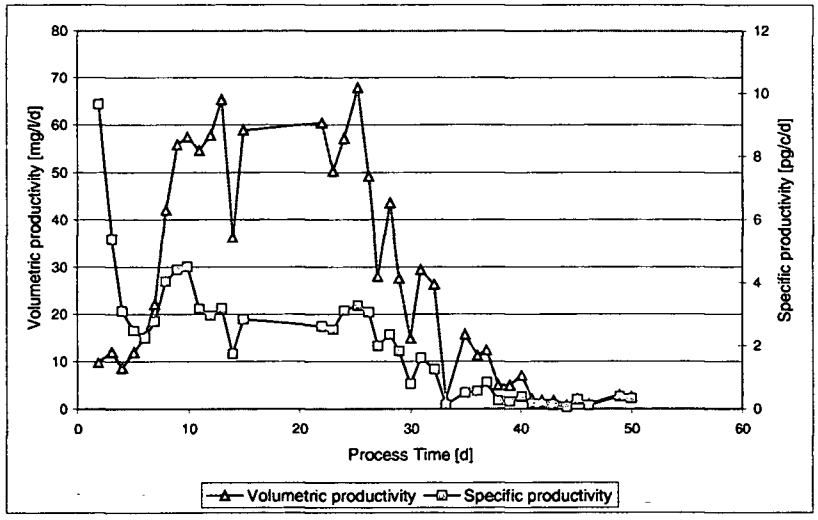


Figure 76: Volumetric and specific productivity of CHO 2F5 on Cytoline I

The reduction of the volumetric productivity is until the cell detachment on day 43 mainly caused by the decrease of the specific productivity. As depicted in Figure 76 it was at already rather low 3 – 4 $pg*c^{-1}*d^{-1}$ until day 27 and hence reached roughly half the value of suspension culture. The temperature reduction on day 23 did not increase the specific productivity as it was the case for CHO 4E10 on Cytoline I. After day 26 it continually declined and finally after the increase of the glucose concentration on day 31 it dropped to values around 0.3 $pg*c^{-1}*d^{-1}$. As shown in Figure 76 it after day 33 practically no antibody is produced any longer.

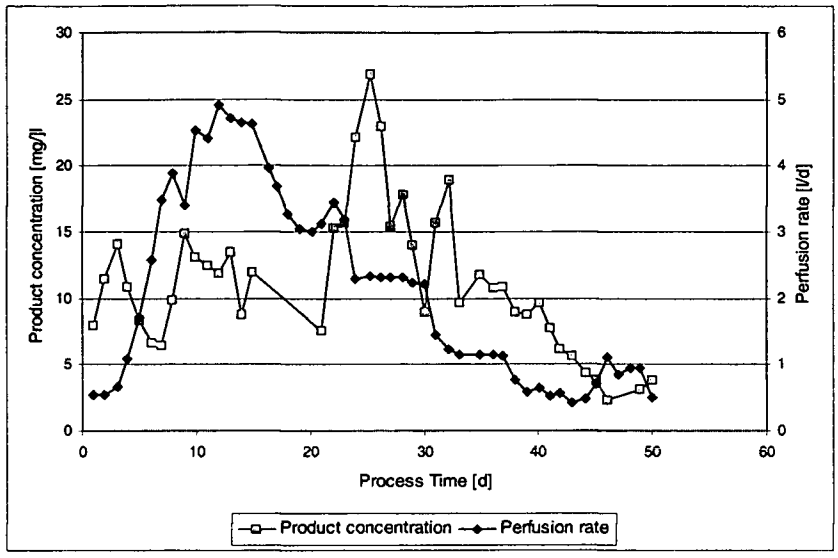


Figure 77: Product concentration and perfusion during CHO 2F5 cultivation on Cytoline I

Due to the low glucose concentration in the medium of only 3.1 g/l the perfusion rates during the first two weeks were extremely high and reached a maximum of 5 RV/d on day 12. In Figure 78 it is shown that the residual glucose concentration is lowered from 1 gl until day 15 to 0.5 g/l. This resulted in an about 30 % reduction of the perfusion rate as shown in Figure 77. A further decrease was then achieved by reducing the cultivation temperature from 37 to 33°C on day 23 which led to a significant increase in product concentration from 15 to 25 mg/l. The cell specific perfusion was halved by the combination of temperature decrease and lowered residual glucose level and was finally brought down to 25% of the value on day 12 by increasing glucose concentration in the medium. However, the rise in product concentration was not a lasting effect and especially after day 30 the drop in specific productivity lead to a decline in product concentration which can be seen in Figure 77.

In Figure 78 the development of the lactate and residual glucose concentration during the FBR culture is shown. During the first two weeks the residual glucose concentration was kept at about 1 g/l which resulted in a corresponding lactate concentration of about 1.5 to 1.7 g/l. When the residual glucose concentration was lowered to 0.5 g/l the lactate concentration increased to 2 g/l. After the glucose concentration in the medium was increased from 3.1 to 4.7 g/l on day 31 the lactate concentration of course rose sharply and reached a maximum of 4.8 g/l on day 43. The glucose concentration was then lowered to 4 g/l again at a residual concentration of about 0.7 g/l which led to a corresponding lactate concentration of 3 g/l. The glucose to lactate conversion rate remained at about 80% throughout the cultivation. It was neither significantly changed by increasing the glucose concentration in the medium nor by the temperature change.

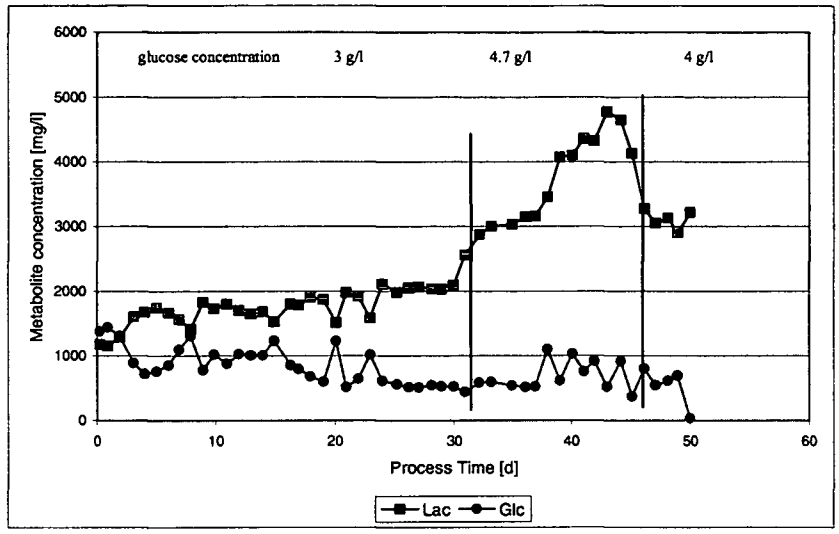


Figure 78: Lactate and residual glucose concentration during CHO 2F5 culture on Cytoline I

As the previous cell lines also the CHO 2F5 cells were cultivated on Cytopore I. In a first attempt a STR was inoculated with cells cultivated in the backup spinner which was run parallel to the FBR fermentation. At this point the cells in the backup spinner had been cultivated for 60 days and still showed normal growth rate and specific productivity. The cells were cultivated as repeated batches to get data about their performance in suspension culture. The cultivation medium was DMEM/Ham's without Pluronic F68. During the first batch viability was around 95% however it dropped to about 90% during the second batch. Pluronic F68 was consequently added to a concentration of 0.1%. Although the cell concentration at the end of the batches remained at about 2 to $2.5*10^6$ cells/ml it took until the end of the third batch to increase the viability to 95% again. After 6 batches Cytopore I carrier were added at a oncentration of 2g/l. However the fermentation had to be ended on the same day due to technical problems.

As shown in Figure 79 the product concentration at the end of the batch is in the same range of 10 to 15 mg/l for the batches 2, 3 and 5. In batch 4 the reactor was passaged at a higher residual glucose level therefore less product was formed. However product concentration in the first batch with 25 mg/l was significantly higher while the residual glucose concentration was almost the same as in the batches 2 and 3.

The development of the specific productivity during the repeated batches is shown in Figure 80. It was in the range of 2 – 3 $pg*c^{-1}*d^{-1}$ and hence on the same level as during the first 4 weeks in the FBR. Nonetheless cells in the STR reached only about half the specific productivity of the first spinner flask batch cultivation (see Figure 74).

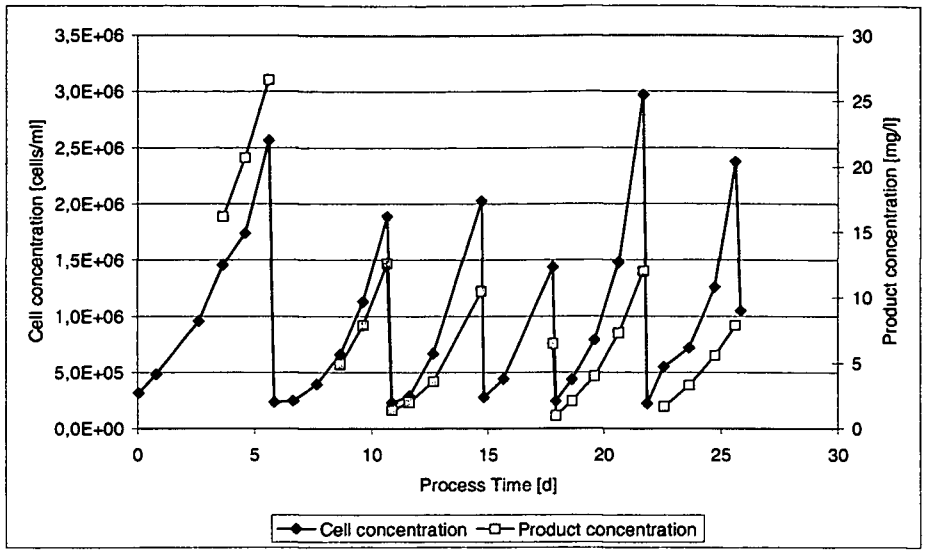


Figure 79: Cell growth of CHO 2F5 in suspension

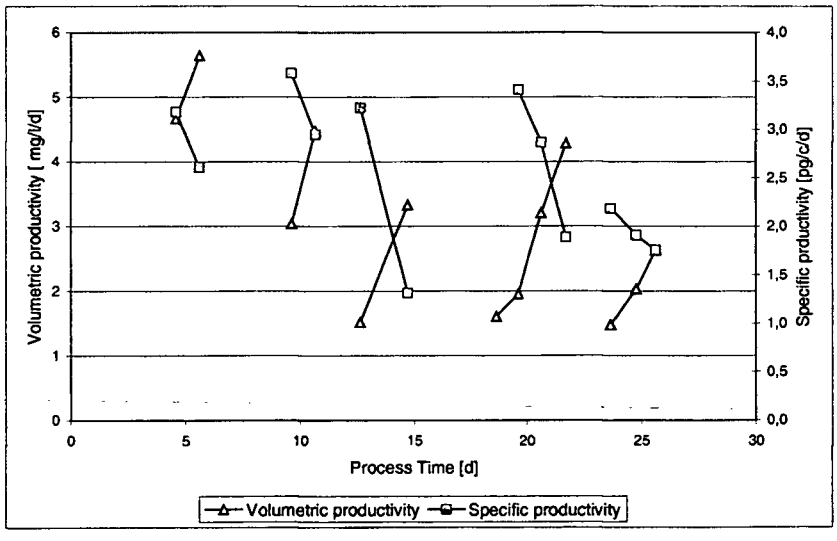


Figure 80: Volumetric and specific productivity of CHO 2F5 in suspension

Of the tested cell lines the inoculum preparation was easiest with CHO 2F5 cells. The comparison of cell performance in different fermentation systems was therefore continued using this cell line. As the DMEM/Ham's medium has only a glucose concentration of 3.1g/l high perfusion rates are required.The glucose concentration was therefore increased to 4g/l. According to the spent media analysis from the previous fermentations also the concentration of the amino acids glutamine, asparagine, aspartic acid, glutamic acid, isoleucine, methionine and tryptophane was increased as shown in 5.1. During suspension culture the medium was supplemented with 0.1% Pluronic F68 and 250µM ferric citrate. While the cells were grown on microcarriers the same medium without pluronic and a ferric citrate concentration of only 100µM was used to facilitate cell attachment.
The cells were thawed, cultivated in a tissue culture flask and after 2 passages transferred to a spinner flask. Inoculum for the FBR and the STR fermentation was generated from the same ampoule of the working cell bank with the difference that the inoculum for the STR was prepared about 1 month after inoculation of the FBR.

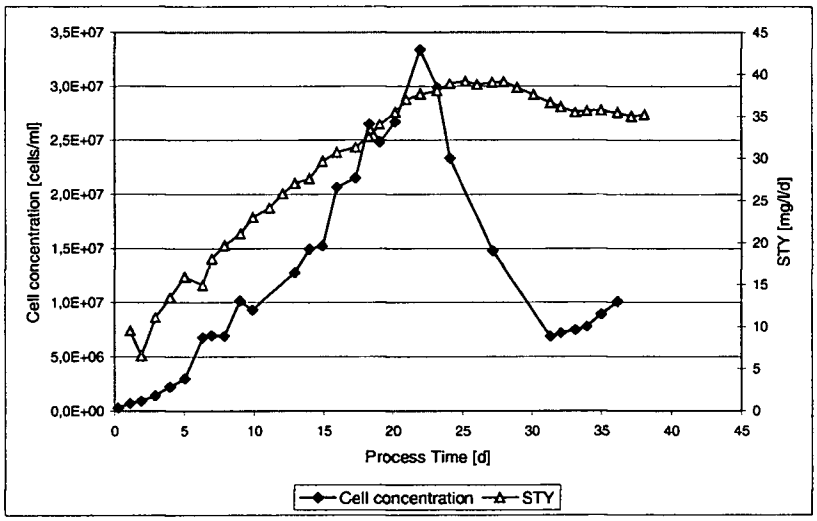


Figure 81: Cell growth and space time yield during cultivation of CHO 2F5 on Cytoline I

As shown in Figure 81 the cells grew quite steadiliy in the FBR. The medium growth rate during the first 3 weeks was 0,22/d which is about half the growth rate reached during routine culture in suspension. A cell concentration of $2.1*10^7$ cells/ml was reached after two weeks and on day 22 the cells had finally grown to their maximum number of $3.3*10^7$ cells/ml. The cell count in the supernatant increased in parallel with the cell growth on the carriers. Up to a total cell concentration of $8.3*10^6$ cells/ml the corresponding cell count in the supernatant was about 1E+05 cells/ml, when $1.6*10^7$ cells/ml had been reached the concentration in the supernatant had increased to 3E+05 cells/ml and finally at a total cell concentration of $3.3*10^7$ cells/ml it was 5 to 6E+05 cells/ml. This rather high cell concentration in the supernatant led to fouling of the distributor plate which is located under the carrier bed and designed to produce a uniform flow that keeps the carriers in suspension. Beginning on day 21 some fluidisation problems were noted. The stirring direction was then changed periodically to reverse the flow through the distributor plate and thus unblock it. The changes in stirring direction were however too slow to create a significant reversed flow through the plate before the carriers had settled and blocked the plate again. Consequently the fluidisation of the carrier bed was dissatisfying between day 23 and 32. High stirring speeds were necessary to get a minimum of carrier movement and oxygen supply for the cells. The resulting shearforces led to cell detachment as can be seen in Figure 81. Until day 32 the cell concentration had

been reduced to $6.7*10^6$ cells/ml. Due to the cell detachment from the carriers the cell concentration in the supernatant increased to $9.5*10^6$ cells/ml The stirring motor was then adjusted to enable a quicker change of the stirring direction and the distributor plate thus unblocked. The stirring speed was reduced from 4 to 600 to 200 rpm and the cell concentration started to increase again from $6.7*10^6$ to $1*10^7$ cells/ml carrier on day 36. While the cell concentration in the supernatant was reduced to $3.5*10^5$ cells/ml.

In Figure 81 the development of the space time yield during the FBR fermentation is shown. It followed the increasing cell number during the first three weeks reaching a maximum of 40 $mg*l^{-1}*d^{-1}$ on day 21 however no further increase occurred due to the decline of the cell concentration which was caused by the fluidisation problems. Figure 82 shows the development of the volumetric productivity which reached a maximum of about 50 to 60 mg/l/d between the days 15 and 25. Afterwards it declined in parallel with the cell concentration and then increased again after proper fluidisation of the carrier bed was reestablished.

The specific productivity is interestingly not significantly influenced by the fluidisation problems. As shown in Figure 82 a major reduction from about 10 to 4 $pg*c^{-1}*d^{-1}$ occured during the first 10 days after inoculation. The specific productivity was then further reduced to 1.5 – 2 $pg*c^{-1}*d^{-1}$ on day 25. The reduction until day 10 happened while the cells were still in exponential growth phase and the cell concentration was around $1*10^7$ cells/ml which means that the carriers had only about 30% of their maximum cell load. As the cells continued to grow and tripled their concentration until day 22 a nutrient limitation seems unlikely during this phase. Amino acid analysis showed low levels of aspartic acid and glutamic acid at the end of the fermentations this however happened after day 25 when the perfusion was reduced. Apart from oxygen limitation which is unlikely due to the low cell number it is not clear what caused this decline of the specific productivity.

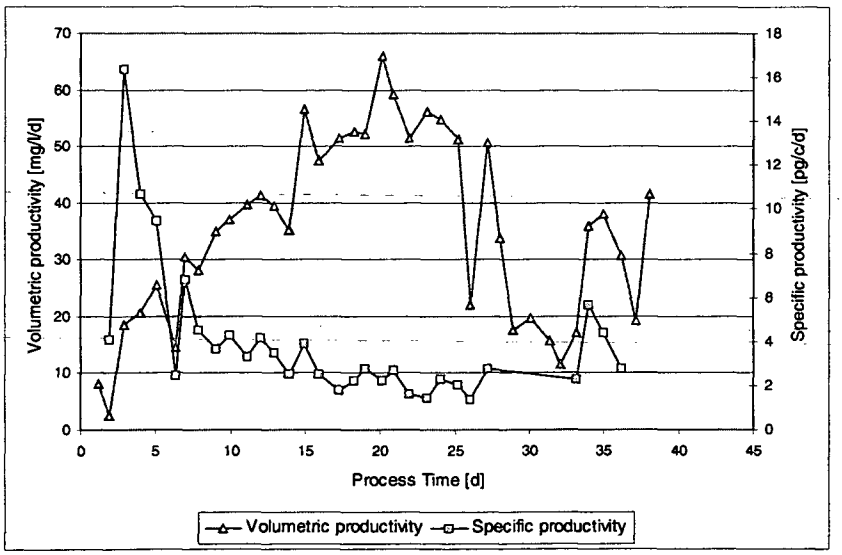


Figure 82: Specific and volumetric productivity of CHO 2F5 on Cytoline I

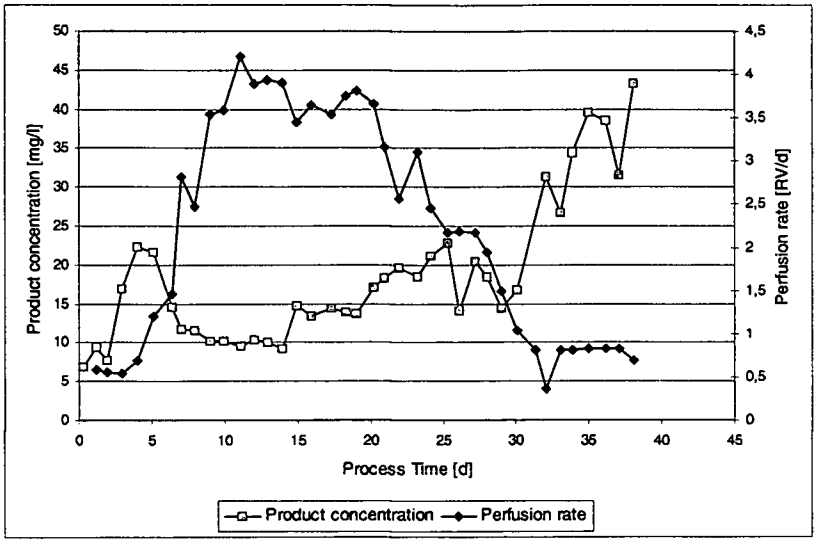


Figure 83: Product concentration and perfusion rate during CHO 2F5 cultivation of Cytoline I

During the first 3 weeks the cell concentration increased steadily. As shown in Figure 84 the residual glucose level was kept at 1.5g/l to avoid nutrient limitations. The perfusion rate was therefore rather high reaching a maximum of 4 RV/d on day 11. This resulted in low product concentrations in the range of 10 to 15 mg/l during the first 3 weeks of the fermentation. In Figure 83 it is shown that the perfusion rate was markedly reduced from day 20 to day 32 which stabilised the product concentration despite the declining volumetric productivity. After the carrier bed was properly fluidised on day 32 the perfusion rate was adjusted to yield a residual glucose level of 0.5 g/l which resulted in a sharp increase of the product concentration from 20 to about 40mg/l.

In Figure 84 the residual glucose and lactate concentrations throughout the fluidised bed fermentation are shown. While the residual glucose concentration was kept at 1.5g/l the resulting lactate concentration was about 2g/l. When the glucose level was lowered to 1g/l the lactate concentration increased to about 2.5g/l. Finally after the refluidisation of the carrier bed the residual glucose concentration was further reduced to 0.5g/l which resulted in a lactate concentration of about 2.8g/l. Cells still grew at this glucose concentration so they seem to tolerate a lactate concentration of at least 3g/l however during the last two days of the fermentation the two amino acids glutamic and aspartic acid reached very low concentrations of only 10 mg/l (data not shown).

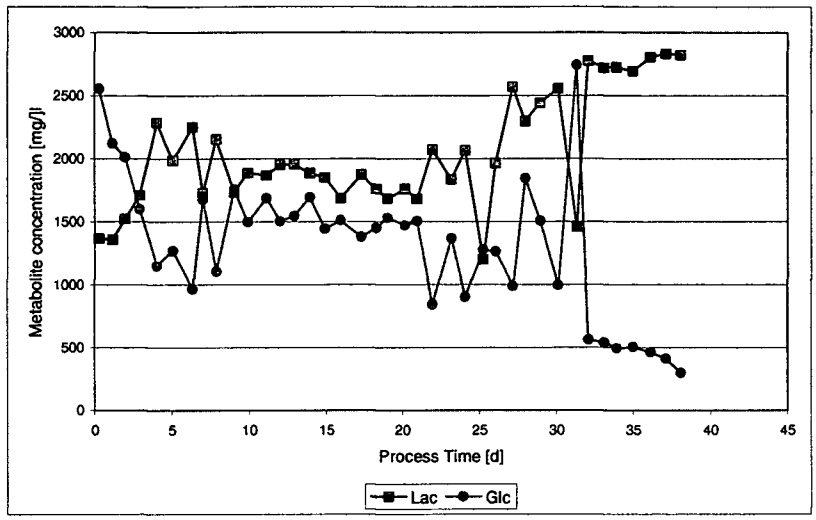


Figure 84: Glucose and lactate concentration during CHO 2F5 cultivation on Cytoline I

For the cultivation on Cytopore I carriers the CHO 2F5 cells were first cultivated in a STR in repeated batch mode to generate inoculum for the carrier fermentation. As shown in Figure 85 the cell count during suspension culture at the end of the batch reached about $1.7*10^6$ cells/ml. The cells were cultivated in medium supplemented with 0.1% Pluronic F68 and in contrast to the previous STR fermentation cell viability was always higher than 96% during suspension culture. After the second batch Cytopore carrier were added to a concentration of 2g/l and almost 90% of the cells attached to the carriers within 7 h. During cell cultivation on Cytopore I growth medium without Pluronic F68 and with a ferric citrate concentration of 100µM was used. In Figure 85 it can be seen that the cells grew fast on the carriers and reached a cell concentration of about $6.5*10^6$ cells/ml on day 15. The average growth rate between day 9 and 15 was 0.3 which is about 50% higher than on the Cytoline carriers. Due to the high cell number the aeration had to be switched from a 1 mm tube connector to a microsparger with a pore size of 0.2µm on day 14. The resulting small oxygen bubbles attached to the Cytopore carriers and transported them into a foam layer thus removing also the cells from suspension. Consequently the cell concentration was halved on the following day. The sparger was therefore switched off and oxygenation done using the tube connector. The cellcount recovered after the foam had dissolved but a high oxygen flow of about 2 vvh was necessarry to keep the setpoint of 50% air saturation. Cell concentration then stabilised at about $8*10^6$ cells/ml until day 24. It then increased to $9*10^6$ cells/ml on day 26. At this point the oxygen demand could not be met any longer and the pO_2 decreased to 30% air saturation. It was then tried to increase the product concentration as much as possible therefore the residual glucose concentration was reduced to 0.25g/l which led to cell detachment and the process was ended on day 28.

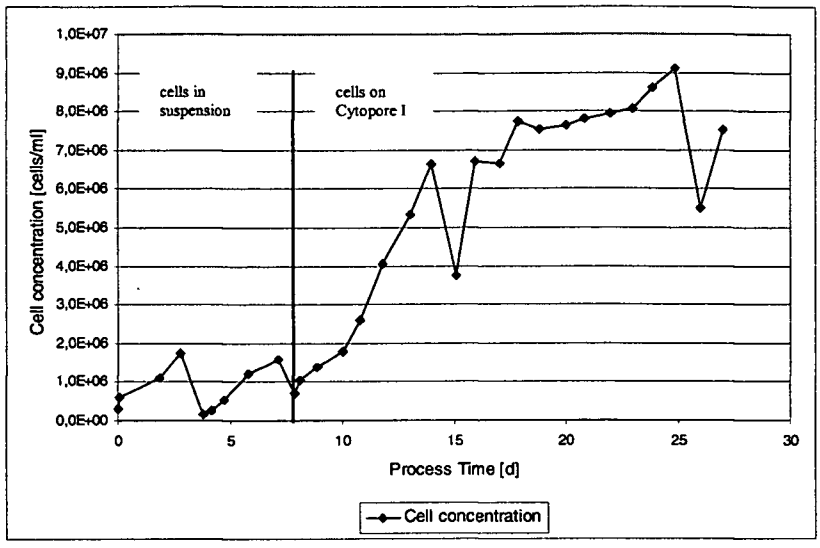


Figure 85: Cell growth of CHO 2F5 in suspension and on Cytopore I

The volumetric productivity increased in parallel with the cell count as shown in Figure 86. There are some fluctuations due to inconsistent ELISA results but it is shown that the maximum volumetric productivity was in the range of 40 to 50 $mg*l^{-1}*d^{-1}$ reached at day 22 and 28.

In Figure 86 the development of the specific productivity during suspension culture and cultivation on Cytopore I is shown. There are rather large variations between 4 and 9 $pg*c^{-1}*d^{-1}$ however there was no difference between cells growing in suspension and on carriers. Also no decline during the 3 weeks of cultivation on Cytopore I was visible. The mean value was 5.6 $pg*c^{-1}*d^{-1}$ which was in the range achieved during routine culture in suspension as shown in Figure 74. In Figure 87 it can be seen that the perfusion rates increased rapidly from day 9 to day 14 reaching a maximum of 1 RV/d. Due to the high perfusion rate product concentration rose only from 20 to 40 mg/l despite the pronounced increase of the volumetric productivity. On day 15 the glucose concentration in the medium was increased from 4 to 5g/l and on day 18 the residual glucose concentration was lowered from 1.5g/l to 0.5g/l. As a result the perfusion rate dropped to about 0.5 RV/d and due to the high volumetric productivity the product concentration was almost tripled to more than 100 mg/l as shown in Figure 87.

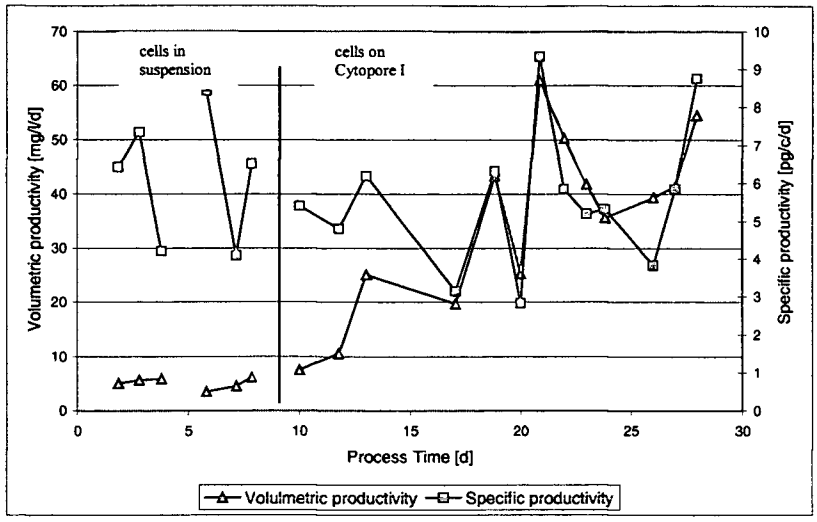


Figure 86: Volumetric and specific productivity of CHO 2F5 in suspension and on Cytopore I

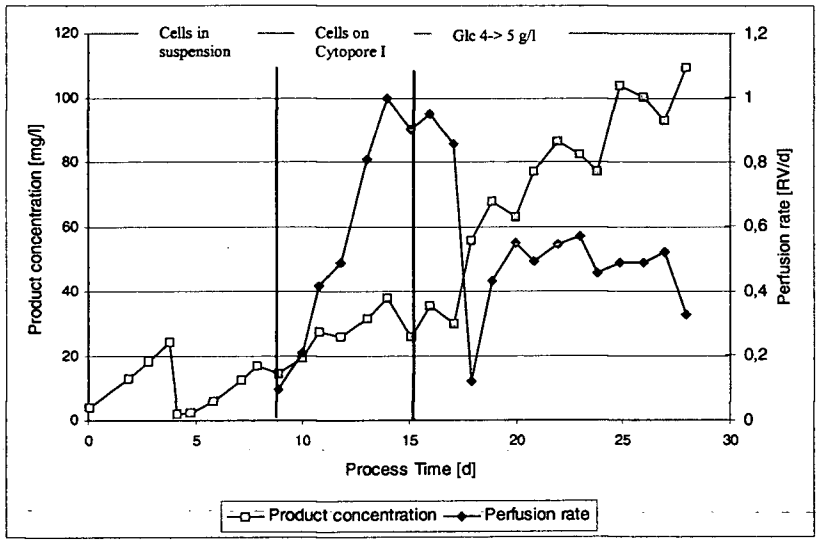


Figure 87: Product concentration and perfusion rate during CHO 2F5 culture in suspension and on Cytopore I

After the addition of the carriers the residual glucose concentration was kept above 1.5 g/l until day 15 which resulted in a lactate concentration of about 2g/l. The glucose concentration in the medium was then increased from 4 to 5g/l which at almost constant perfusion rate led to an increase in residual glucose concentration to more than 2g/l as shown in Figure 88. On day 18 the residual glucose concentration was lowered to 0.5g/l to reduce the perfusion rate. The corresponding lactate concentration stabilised at about 2.5g/l. By increasing glucose concentration and lowering residual glucose the cell specific perfusion was reduced from about 200 pl/c/d between day 9 and 17 to about 50 - 70 pl/c/d from day 18 to 28. The specific glucose consumption was reduced from 540 to about 300 $pg*c^{-1}*d^{-1}$ and the specific lactate production was reduced from 480 to 150 $pg*c^{-1}*d^{-1}$. Consequently also the glucose/lactate conversion rate declined from 85 to 50%.

At least during the last two days of the fermentation glutamic and aspartic acid reached very low concentrations of about 10 mg/l. Also the asparagine concentration of 20 mg/l and the glutamine concentration of about 50 mg/l

might already have reached limiting levels which could be the reason for the declining cell number on day 27 and 28.

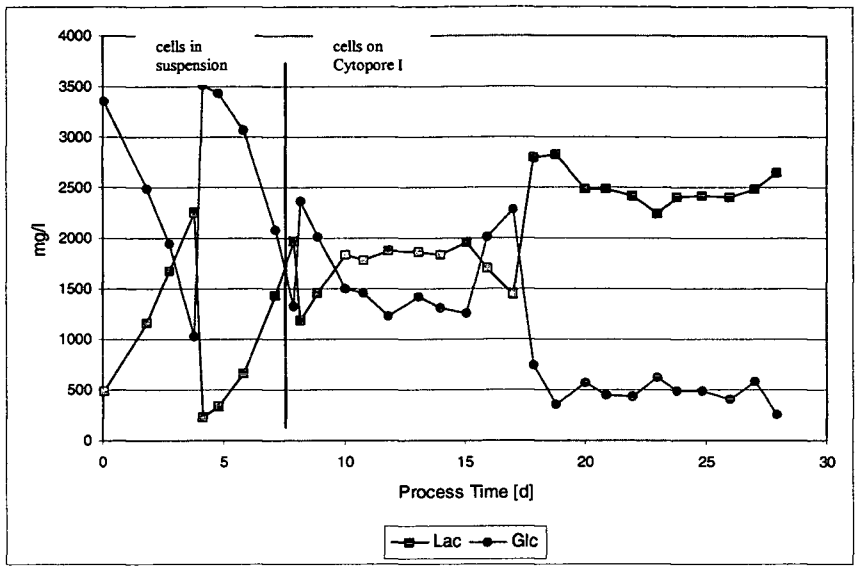


Figure 88: Glucose and lactate concentration during CHO 2F5 cultivation in suspension and on Cytopore I

6.3.5 Experiments to Optimise Oxygenation in Cytopore I Fermentations

During cell cultivation on Cytopore carriers a trade off between efficient oxygen transfer and critical bubble size had to be found. On the one hand the cell concentration is rather high reaching values of more than $1*10^7$ cells/ml which requires an efficient way of oxygen transfer and hence a large bubble surface. The small bubbles, however, which are required to obtain the large surface area, frequently attach to the carriers and transport them to the medium surface where they create a stable foam layer and trap the carriers. Larger gas bubbles rise faster and burst more quickly after reaching the medium surface.

In all fermentations aeration was done using pure oxygen. Applying pressure to increase oxygen solubility was not feasible in the laboratory reactors. The only adjustable parameter therefore was the size of the gas bubbles. A series of experiments with different sparger variants was conducted to find an oxygenation device that was more efficient then the so far used 1 mm tube connector but created much less foaming than the 0.2 µm sparger.

Besides the tube connector a ringsparger with 14 x 1 mm holes, 3 sparger with ceramic frits and the 0.2 µm sparger were tested. The ceramic frits had an undefined pore size but varied in their surface area. The smallest one had a surface area of about 1.5 cm^2, frit 2 had about 3 cm^2 and finally the largest one had a surface area of about 4.5 cm^2. All devices were operated at a gas flow rate of 50 ml/min and 200 ml/min the results are shown in Table 19.

	kLa at 50 ml/min [1/s]	% tube conn.	kLa at 200 ml/min [1/s]	% tube conn.
tube conn. 1mm	8,75E-05	100	1,36E-04	100
ringsp. 14x1mm	9,94E-05	114	2,79E-04	205
sparger frit 1	2,18E-04	249	6,67E-04	490
sparger frit 2	1,05E-03	1200	2,14E-03	1574
sparger frit 3	1,33E-03	1520	2,51E-03	1846
sparger 0.2 µm	1,03E-03	1177	2,81E-03	2066

Table 19: k_La values of different oxygenation methods

Frit 1 had a significantly smaller surface area than the other two ceramic spargers and the 0.2 µm sparger. However, the bubble size is in the same range and it therefore cannot be used in fermentations with Cytopore carriers. The ringsparger had about the double efficency compared to the tube connector at the higher flow rate of 200 ml/min. The bubble size is much larger compared to the frits and the microsparger therefore no stable foam layer is created. For aeration during the following STR fermentations accordingly the ringsparger was used.

6.3.6 Increasing cell- and product concentrations in CHO 2F5 cultivations

The objective of the following bioreactor cultivations was to increase the cell concentration using the optimised oxygenation method and to increase the product concentration by applying an optimised cultivation medium and therefore achieving lower perfusion rates. Furthermore it was also tested if the specific productivity of CHO 2F5 cells can be increased by adding sodium butyrate to the cultivation medium.

For the following CHO 2F5 fermentation cells were thawed and grown in a tissue culture flask. After the second passage they were transferred to a spinner flask. The medium for fermentation was the same as used for the previous CHO 2F5 experiments however the concentrations of the amino acids glutamine, serine and lysine were further increased to prevent nutrient limitation at low residual glucose levels as indicated in 5.1. The STR was directly inoculated with cells grown in spinner flasks.

In Figure 85 the development of the cell concentration and the space time yield is shown. The cells grew with a medium rate of 0.3/d during the first two weeks an reached a concentration of $1.17*10^7$ cells/ml on day 15. At this point the stirring speed was increased from 120 to 200 rpm to break up some carrier aggregates that had formed. The higher stirring speed was probably the reason for the cell loss that occurred in the following days. Additionally gas bubbles were drawn into the carrier retention and obstructed the settling of the carriers wich led to some carrier loss. On day 19 the stirring speed was therefore reduced again to 120 rpm. On day 22 the glucose concentration in the medium was increased from 4 to 5 g/l. On day 25 the cell number started to increase again it reached a concentration of 1.2 to $1.3*10^7$ cells/ml between day 29 and 41 and then decreased to about $8*10^6$ cells/ml on day 50. To enhance specific productivity sodium butyrate was added to the medium. The starting concentration on day 31 was 75 µM it was then doubled to 150 µM on day 35 which led to a reduction in oygen demand. On day 41 the sodium butyrate concentration was increased to 250 µM which might be the reason for the decline of cell concentration to $8*10^6$ cells/ml on day 44. Nonetheless the sodium butyrate concentrations

were further increased to 500 µM on day 44 and 1 mM on day 49 to test the effect on specific productivity. The increase in butyrate concentration is shown in Figure 90.

The development of the volumetric productivity is shown in the same graph it reaches a maximum of about 65 mg/l/d on the days 15 and 16 and then ranges between 40 and 50 mg/l/d from day 17 to 50.

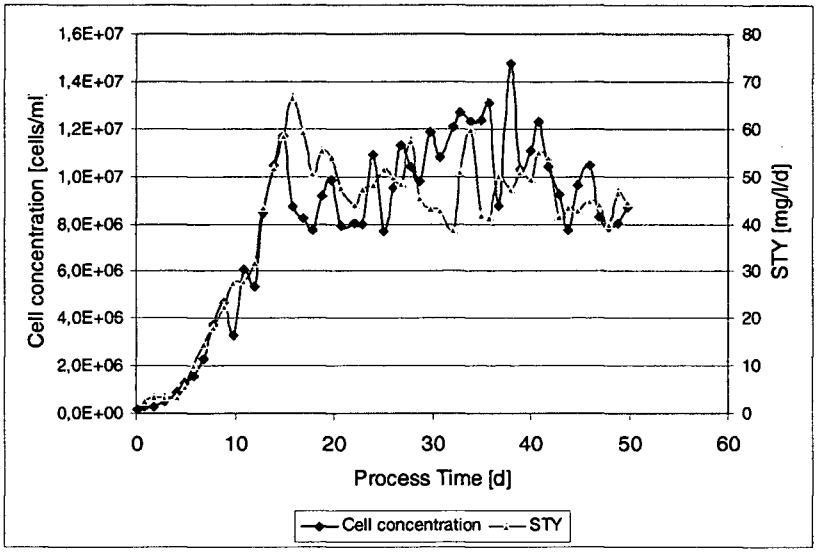


Figure 89: Cell growth and space time yield during CHO 2F5 cultivation on Cytopore I

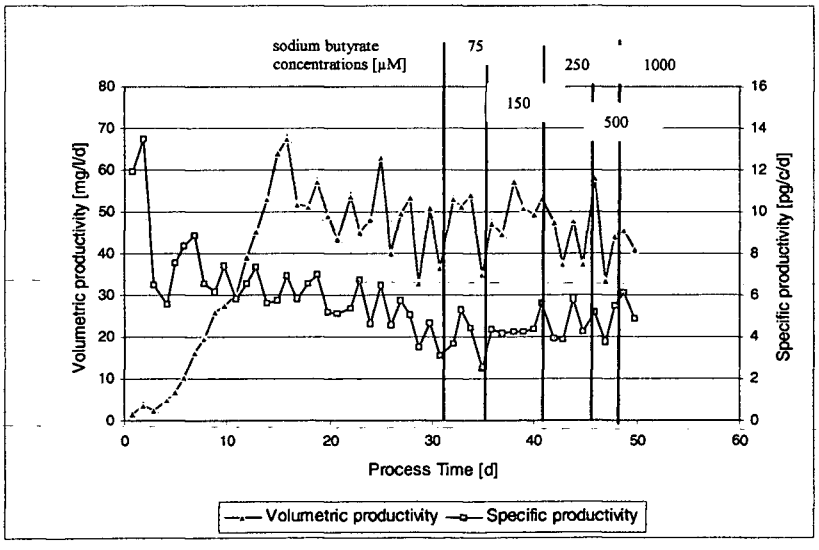


Figure 90: Specific and volumetric productivity of CHO 2F5 on Cytopore I

As shown in Figure 90 the specific productivity was in the range of 6 to 8 $pg*c^{-1}*d^{-1}$ during the first 3 weeks. The high values of 12 and 13.5 $pg*c^{-1}*d^{-1}$ on day 2 and 3 are probably artefacts. After day 20 the specific productivity declined to about 4 $pg*c^{-1}*d^{-1}$ on day 30. From day 32 to 40 it stabilised at a level of about 4 $pg*c^{-1}*d^{-1}$ and then increased to a medium value of 5 $pg*c^{-1}*d^{-1}$ between day 41 and 50. The decline after day 20 probably occurred due to the reduced perfusion rate which led to a depletion of glutamic acid between day 28 and 32 although glutamine was still present at concentrations of more than 100 mg/l as shown in Figure 91. After the addition of sodium butyrate on day 31 the consumption rates for glucose and amino acids decreased significantly

and the concentrations of glutamine and glutamate in the spent medium rose although the residual glucose concentration was kept at 0.5 mg/l. It is therefore not quite clear if the stabilisation and increase of the specific productivity was due to the effect of the sodium butyrate itself or because it changed cell metabolism and reduced consumption of glutamate. Judged from the volumetric productivity however a sodium butyrate concentration of more than 250 µM was not beneficial as it led to cell detachment and thus caused lower yields despite the increased specific productivity.

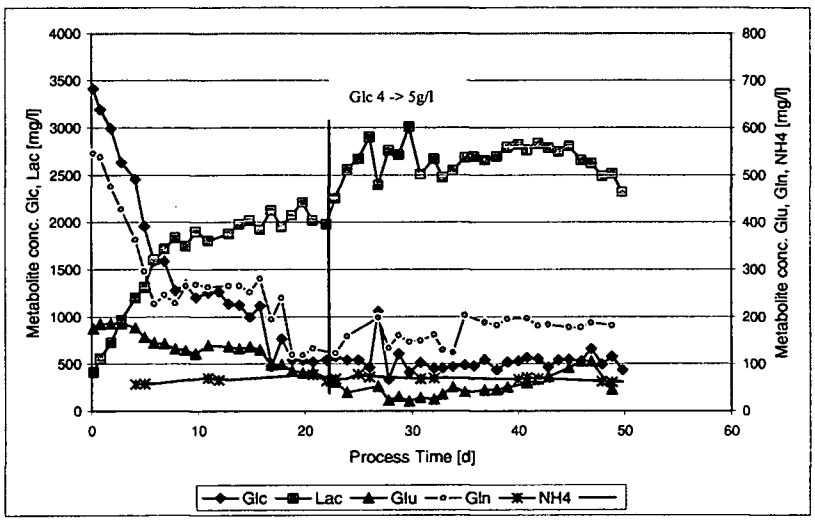


Figure 91: Metabolite concentrations during CHO 2F5 cultivation on Cytopore I

In Figure 91 the development of selected metabolite concentrations is shown. With declining residual glucose concentration the lactate concentration rose and reached a level of 2 g/l at a residual glucose concentration of 1 g/l. On day 15 the residual glucose concentration was reduced from 1 g/l to 0.5 g/l which caused only a slight increase in the lactate concentration to about 2.2 g/l. However, increasing the glucose concentration in the medium from 4 to 5 g/l on day 22 had a more pronounced effect and resulted in a lactate concentration of about 2.8 g/l. As shown in Figure 91 there was a slight reduction of the lactate concentration between day 31 and 34. It was probably caused by changes in cell metabolism either triggered by the addition of sodium butyrate on day 31 or by the lack of glutamate between day 28 and 34. The glutamine concentration fell quickly from about 550 mg/l on day 1 to 250 mg/l on day 6. It then remained at this level while the residual glucose concentration was kept around 1 g/l. The reduction of the latter to 0.5 g/l on day 15 caused the glutamine concentration to drop to 100 mg/l. Interestingly supplementing the medium with an additional gram of glucose did not result in a further decline of the glutamine concentration but rather caused a slight increase to about 150 mg/l. After the increase of sodium butyrate concentration to 150 µM on day 35 it increased to about 200 mg/l at a constant residual glucose level of 500 mg/l. The cell concentration was around $1.2*10^7$ cells/ml between the days 30 and 41 as also the perfusion rate was kept constant during this time the increasing residual levels of glutamine and glutamate were caused by a reduction in the specific consumption rates. The residual glutamine concentration was neither influenced by the subsequent rising of the sodium butyrate concentrations on days 41, 44 and 48 nor by the declining cell count after day 41. The development of the residual glutamate concentration shows some significant differences to the glutamine concentration. During the first two weeks of the process it declined only moderately from about 190 to 140 mg/l although the residual glucose concentration dropped from 3.5 to 1 g/l. It then rea-

ched a level of about 80 mg/l when the residual glucose concentration was lowered to 500 mg/l. In contrast to the glutamine concentration it did not stablise at this level but dropped to very low concentrations of only 20 to 30 mg/l after the increase of the glucose concentration in the medium on day 22. Three days after the addition of sodium butyrate the residual glutamate concentration rose to about 50 mg/l. Rising the sodium butyrate concnentration from 75 to150 µM did not significantly change the specific consumption rates and the residual concentration remained at 50 mg/l. After day 41 the increase in sodium butyrate concentration to 250 µM caused cell detachment which probably in combination with the changed cell metabolism led to a rise of the residual glutamate concentration to 100 mg/l on the days 45 to 47. The ammonium concentration changed only moderatly throughout the fermentation. Despite the high glutamine consumption it increased only from 60 mg/l on day 4 to 80 mg/l after the reduciton of the perfusion rate on day 22 (see Figure 91). After the addition of sodium butyrate it decreased to 70 mg/l on day 32 and finally 60 mg/l on day 49.

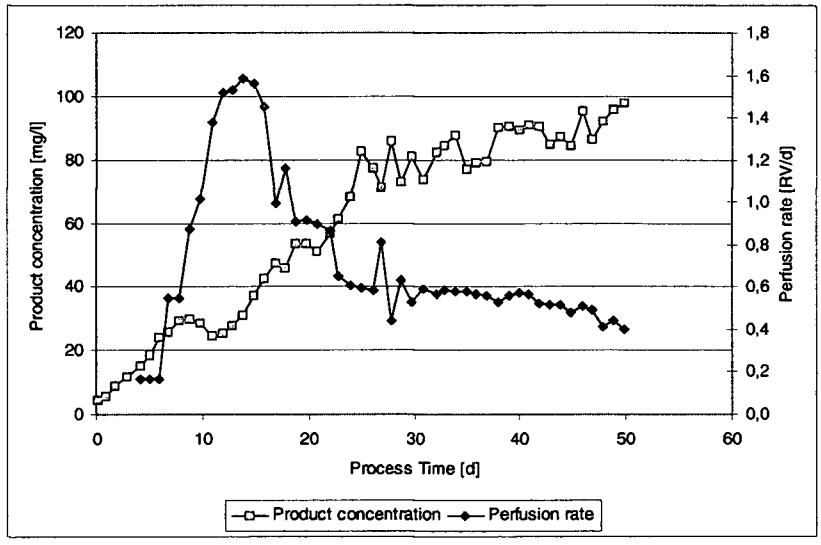


Figure 92:Product concentration and perfusion rate during CHO 2F5 cultivation on Cytopore I

The perfusion rate jumped from 0.2 RV/d on day 6 to 1.6 RV/d on day 14 as shown in Figure 92. It was then reduced to about 1 RV/d by decreasing the residual glucose concentration from 1 g/l to 0.5 g/l which also reduced the specific glucose consumption from about 450 to 300 $pg*c^{-1}*d^{-1}$. When the glucose concentration in the medium was increased from 4 to 5 g/l on day 22 a further drop in the perfusion rate to about 0.6 RV/d was achieved. It then remained at this level until day 41 and was then finally reduced to 0.4 RV/d on day 50 due to the declining cell number. The product concentration increased steadiliy throughout the fermentation. The major effects were gained by reducing the residual glucose concentration to 1 g/l on day 15 which brought the product concentration to about 50 mg/l and as well by the increase of the glucose concentration in the medium from 4 to 5g/l on day 22 which boosted the antibody concentration in the supernatant from 50 to 80 mg/l. Until the end of the fermentation the product concentration was gradually increased to more than 100 mg/l as shown in Figure 92 by continually lowering the perfusion rate after day 41.

CHO 2F5 cells were again cultivated in a FBR to compare the performance of the cells on the two carrier types. The cells for inoculation were derived from the backup spinner run parallel to the preceeding fermentation in the STR. The reactor was inoculated with 1 l of suspension culture, 70% of the cells attached within 7 hours and the starting cell concentration was $3.75*10^5$ cells/ml.

As shown in Figure 94 the cells reached a concentration of about $2*10^7$ cells/ml after 2 weeks. The average growth rate during this period was 0.3/d. The cellcount remained at $2*10^7$ to $2.3*10^7$ cells/ml between day 15 and 40. The residual glucose concentration was then decreased from 500 to 300 mg/l which resulted in a drop of the cell concentration to about $1.8*10^7$ cells/ml and a further decline to $1.5*10^7$ cells/ml until day 53. To further reduce the perfusion rate the residual glucose concentration was lowered to 100 mg/d on day 53 this however resulted in a further drop of the cell concentration to about $1.1*10^7$ cells/ml and due to the decrease in volumetric productivity no increase of the product concentration was achieved. In Figure 94 also the concentration of suspended cells in the supernatant is shown. It is important to keep this cell count low as the cells cause fouling of the reactor's distributor plate and retention sieve. It can be seen that the cell concentration remained at moderate $1*10^5$ to $3*10^5$ cells/ml during the first month of the process. On day 31 the glucose concentration in the medium was increased to 5.5 g/l with a residual glucose level of 500 mg/l. This resulted in low glutamate levels of only 20 to 30 mg/l although glutamine was still present at concentrations of more than 200 mg/l. The lacking glutamate was probably the reason for some cell detachment and the increased suspension cell concentration which reached a level of $6*10^5$ to $7*10^5$ cells/ml between the days 35 and 56. The stirring speed had to be increased from 260 rpm on day 30 to 330 rpm on day 53 however the carrier bed was kept fluidised throughout the process due to periodic changes of the stirring direction which prevented the blocking of the distributor plate.

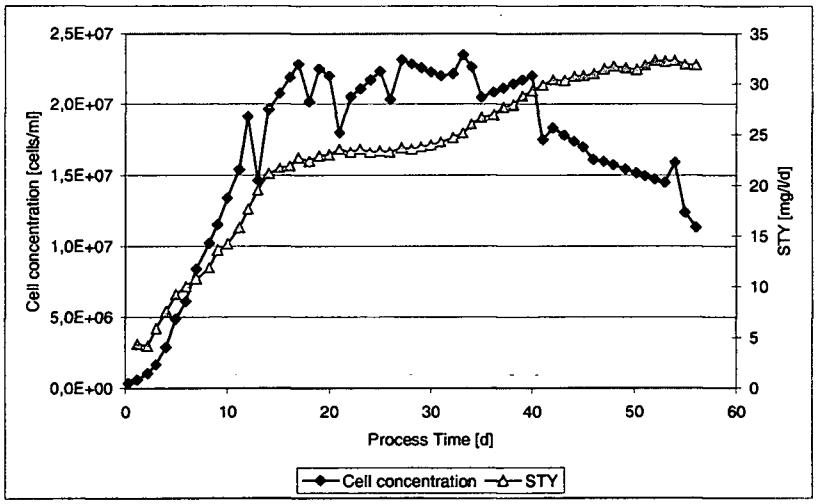


Figure 93: Cell concentration and space time yield during cultivation of CHO 2F5 on Cytoline I

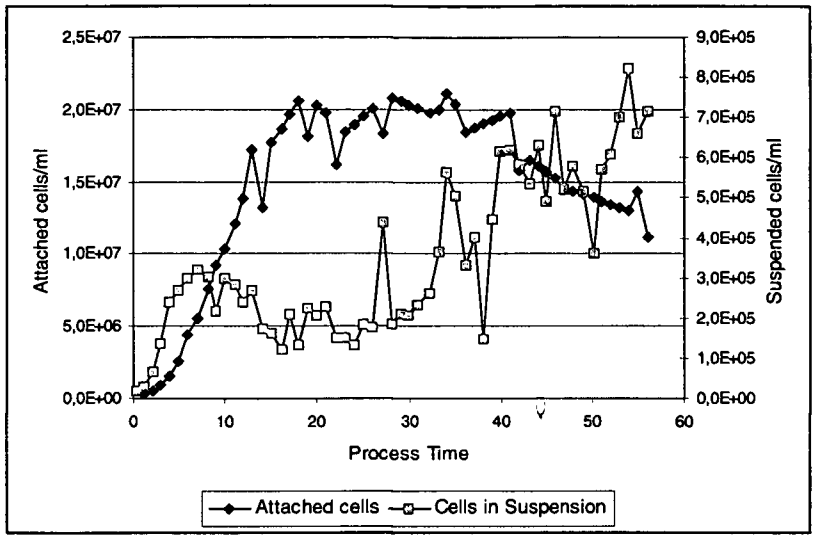


Figure 94: Cell concentration on microcarriers and in suspension during the cultivation of CHO 2F5 on Cytoline I

In Figure 95 the development of specific and volumetric productivity during the FBR fermentation is shown. Until day 4 the specific productivity was in the range of 5 to 7 $pg*c^{-1}*d^{-1}$ which is also the level usually reached in suspension cultivation (see Figure 80). It then dropped to 2 $pg*c^{-1}*d^{-1}$, remained at this value between day 7 and 14 and finally decreased to about 1.5 $pg*c^{-1}*d^{-1}$ between day 15 and 30. Until day 24 when the glucose concentration in the medium was increased from 4 to 5 g/l a nutrient limitation can be excluded at least regarding the amino acids. It is however unlikely that the decrease of the specific productivity happened due to nutrient limitation as the major drop occurred already between day 4 and 7 when only a cell count of about 5E+07 cells/ml was reached and the residual glucose concentration was still at more than 1 g/l. After day 30 the specific productivity increased again and reached levels of about 2.5 to 3 $pg*c^{-1}*d^{-1}$ until day 53 of the process when the residual glucose level was lowered to 100 mg/l. As the glutamate concentration remains below 30 mg/l from day 28 until the end of the cultivation the lack of this amino acid does not seem to be the reason for the decreased specific productivity. After day 30 the perfusion rate was gradually lowered from 1 RV/d to about 0.5 RV/d as shown in Figure 96. This may have led to a better conditioning of the medium and thus caused the increase in specific productivity.

The volumetric productivity reached a level of 35 to 45 $mg*l^{-1}*d^{-1}$ between day 33 and 52 as shown in Figure 95. The cell concentration remained about constant between day 14 and 40. The specific productivity increased from about 1.5 $pg*c^{-1}*d^{-1}$ to 2.5 $pg*c^{-1}*d^{-1}$ on day 34 and was the reason for the rise in the total productivity at this point. The specific productivity then further increased to about 3 $pg*c^{-1}*d^{-1}$ and thus compensated the drop in cell concentration on day 41. The volumetric productivity finally decreased after day 53 when the cell concentration dropped to $1.2*10^7$ cells/ml.

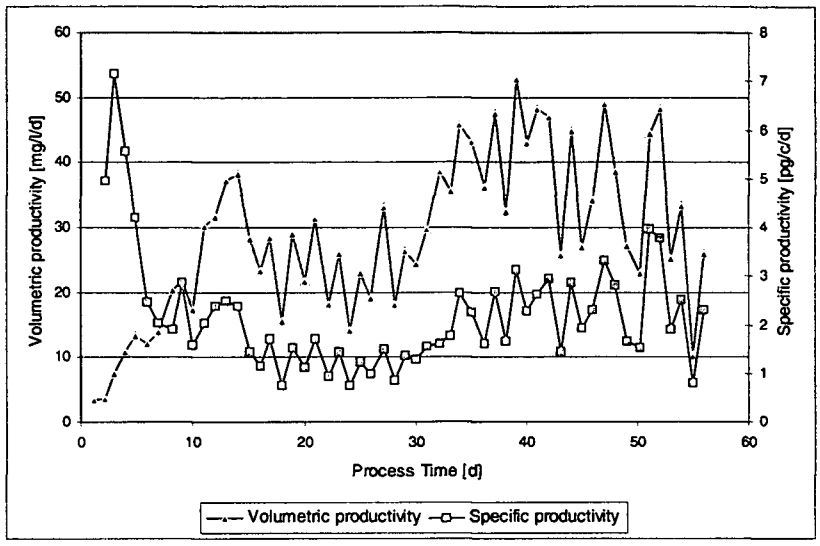


Figure 95: Volumetric and specific productivity of CHO 2F5 on Cytoline I

As shown in Figure 96 the perfusion rate rose steeply from 0.5 to 3.5 RV/d between day 4 and 14. During the process the perfusion rate was then continually lowered by increasing the glucose concentration in the medium and also reducing the residual glucose concentration that was finally pushed down to 150 mg/l on day 56. At the start of the fermentation the glucose concentration in the medium was 4g/l the residual glucose concentration was kept at 1.5 g/l until day 16 when it was lowered to 1 g/l which resulted in a decrease of the perfusion rate from 3.5 to 3 RV/d. On day 20 the residual glucose concentration was reduced to 500 mg/l which resulted in a perfusion rate of about 1.5 RV/d from day 22 to 24 as shown in Figure 96. At the lower residual glucose concentration less than half of the maximum perfusion rate was required although the cell concentration was practically the same. To further reduce the perfusion rate the glucose concentration of the medium was increased from 4 to 5 g/l on day 24. At a residual glucose concentration of 500 mg/l this resulted in a perfusion rate of about 1 RV/d between day 25 and 31. On day 31 the glucose concentration was then increased to 5.5 g/l the cells adapted to the new situation and the specific glucose consumption was slightly reduced from about 170 $pg*c^{-1}*d^{-1}$ on day 31 to about 140 $pg*c^{-1}*d^{-1}$ on day 41. Consequently the perfusion rate was lowered from 1 to 0.7 RV/d. It was then tried to further increase the product concentration by reducing the residual glucose level from 500 to 300 mg/d on day 41 and finally to 100 mg/l on day 53. The perfusion rate was decreased to about 0.5 RV/d until day 53 and finally to 0.4 RV/d on day 56.

Due to the high perfusion rate and the low specific productivity the product concentration was only around 10 mg/ml during the first 3 weeks of the process. After the perfusion rate was reduced below 1.5 RV/d on day 21 the product concentration started to increase. The continuous decline of the perfusion rate in combination with the higher specific productivity after day 31 caused an increase of the product concentration to about 60 mg/l on day 41. Afterwards only a slight increase to about 70 mg/l was achieved by further reducing the perfusion rate on day 53. However the volumetric productivity already slightly declined during this period. The subsequent decline of the perfusion rate to 0.4 RV/d further decreased the volumetric productivity and also led to a lower product concentration due to the reduction in cell concentration.

In Figure 97 the development of the metabolite concentrations during the FBR process is shown. At a glucose concentration of 4 g/l in the medium the residual glucose level was kept at 1.5 g/l until day 17 the corresponding lactate concentration was about 2 g/l. The reduction of the residual glucose concentration to 1 g/l led only to a minimal increase in lactate concentration to 2.2 g/l. Interestingly, the decrease of the glucose concentration to 500 mg/l did not show any effect on the lactate concentration. Rising the glucose concentration in the medium from 4 to 5 g/l on day 24 led to an increase of the lactate concentration to 2.5 g/l from day 25 to day 27. It is not clear what caused the sudden increase of the lactate concentration from day 27 to 28. The lactate level rose from 2.5 to more than 3 g/l although residual glucose concentration and glucose concentration in the medium were kept constant. On day 31 the glucose concentration in the medium was increased from 5 to 5.5 g/l consequently the lactate concentration rose from 3.2 to 3.4 g/l. It then remained at this level until day 41. At that point the perfusion rate was calculated to give only a residual glucose concentration of 300 mg/l however due to the declining cell concentration it lasted until day 50 to actually reach the desired concentration. On day 53 the residual glucose concentration was further reduced and finally reached a level of 150 mg/l on day 56.

The residual glutamate concentration remained rather constant at about 150 mg/l until day 24. When the glucose concentration in the medium was increased to 5 g/l glutamate concentrations decreased to 20 – 30 mg/l however neither cell concentration nor specific productivity were negatively influenced.

The residual glutamine concentration sharply decreased during the first days in parallel with the falling residual glucose concentration. As shown in Figure 97 it then increased again up to the starting concentration of about 450 mg/l until day 17 as long as the residual glucose concentration was kept at 1.5 g/l. The lowering of the residual glucose concentration to 1 and 0.5 g/l caused a decline of the glutamine concentration to about 240 mg/l. Rising the glucose concentration in the medium to 5 g/l hardly influenced the residual glutamine concentration it was however reduced to about 200 mg/l when the glucose concentration was stepped up to 5.5 g/l. The residual glutamine concentration then remained at this level until the residual glucose level was reduced to 300 mg/l on day 41 which caused the glutamine concentration to decrease to 130 mg/l on day 51. Even the further reduction of the residual glucose level to 150 mg/l on day 56 only reduced the glutamine concentration to about 100 mg/l.

Despite the rather high glutamine consumption especially after day 41 the ammonium level increased only moderately. The minimal concentration of about 30 mg/l was of course reached during the phase where the residual glutamine concentration was highest. As shown in Figure 97 this happened between day 10 and 20. With the declining residual glutamine concentration the ammonium concentration continually rose and reached a maximum of about 90 mg/l on day 48.

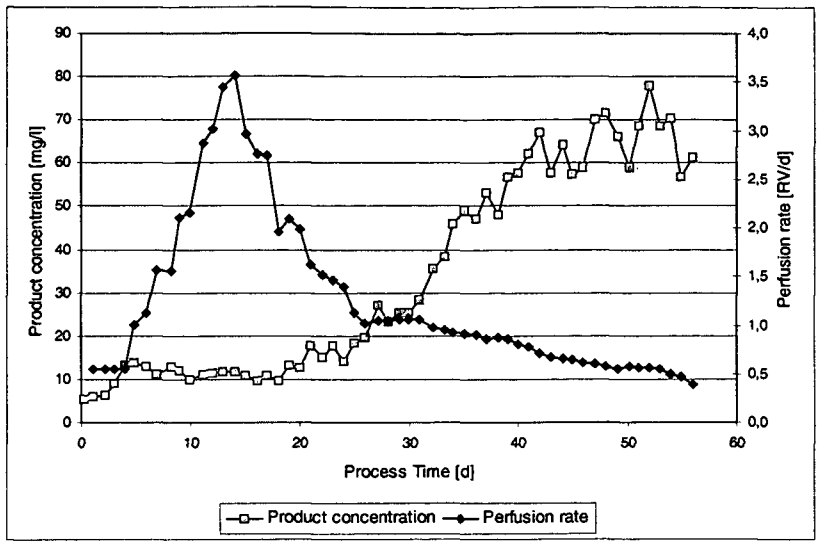


Figure 96: Product concentration and perfusion rate during CHO 2F5 cultivation on Cytoline I

As shown in Figure 98 the specific glucose consumption was more than halved by reducing the residual glucose concentration and increasing the glucose concentration in the medium. On day 16 at a residual glucose concentration of 1.5 g/l it was 300 $pg*c^{-1}*d^{-1}$. On day 24 the residual glucose concentration was 500 mg/l and the specific glucose consumption had declined to 250 $pg*c^{-1}*d^{-1}$. Rising the glucose concentration in the medium to 5 g/l reduced the specific glucose consumption to 170 $pg*c^{-1}*d^{-1}$. The increase of the glucose concentration to 5.5 g/l did not change the specific consumption rate at a residual glucose level of 500 and 300 mg/l however when the residual concentration was reduced to 150 mg/l also the specific consumption rate declined to 130 $pg*c^{-1}*d^{-1}$. The specific lactate production practically followed the development of the glucose consumption. It was reduced from 250 $pg*c^{-1}*d^{-1}$ to 150 $pg*c^{-1}*d^{-1}$ by decreasing the residual glucose concentration from 1.5 to 0.5 g/l. Increasing the glucose concentration in the medium to 5 and finally 5.5 g/l did not have a significant effect on the specific lactate production rate. Only after lowering the residual glucose concentration to 300 mg/l also the specific lactate production rate was reduced to about 120 $pg*c^{-1}*d^{-1}$.

Generally speaking it is of course desirable to keep the specific glucose consumption and lactate production as low as possible. However a compromise has to be found to balance low consumption rates against cell detachment from the carriers. In the case of CHO 2F5 cells the optimum seems to keep the lactate concentration below 3 g/l by using an initial glucose concentration of not more than 5 g/l and to keep the residual glucose level at 0.5 g/l. A lactate concentration of more than 3 g/l caused an increase in the concentration of suspension cells as can be seen in Figure 94 while a residual glucose concentration below 0.5 g/l led to a significant cell detachment as shown in the same graphic.

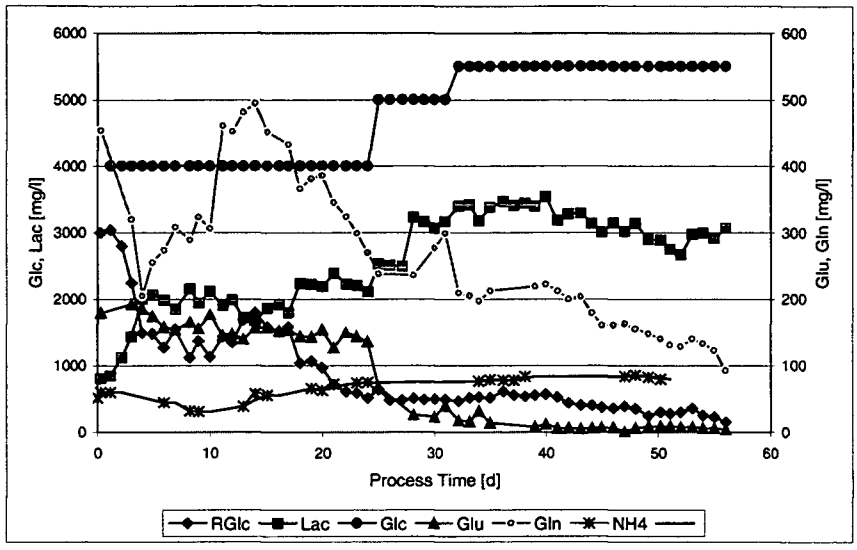


Figure 97: Metabolite concentrations during CHO 2F5 cultivation on Cytoline I

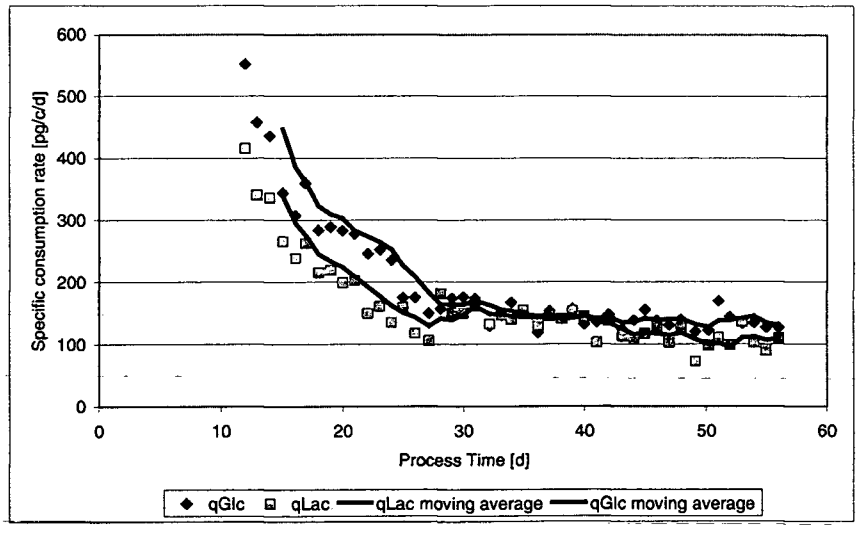


Figure 98: Specific glucose consumption and lactate production of CHO 2F5 on Cytoline I

To compare the cell performance on Cytopore I, Cytoline I and in suspension culture CHO 2F5 cells were also cultivated in a STR in batch and fedbatch mode. As shown in Figure 99 cell concentration in both cultivation modes were around $4*10^6$ cells/ml. The growth rate during exponential phase until day 5 was about 0.4/d. Viability in batch culture decreased quickly after day 6 when glutamine was depleted. The fermentation was ended on day 10 when the viability was down to 20%. In fed batch culture the feed was started on day 5 before the clture ran out of glutamine. The viability dropped below 90% about one day earlier than in the batch culture but declined more slowly afterwards which resulted in an almost 30% increase in the viable cell integral from $1.5*10^7$ to $1.9*10^7$ cells*d*ml^{-1}.

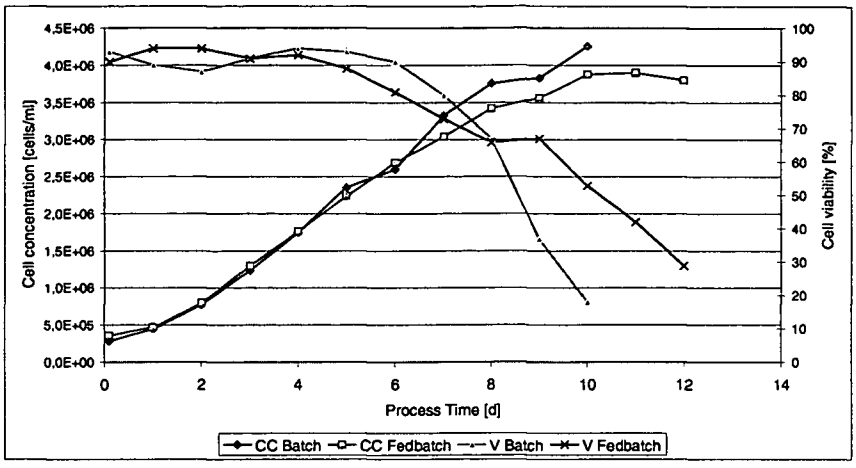


Figure 99: Cell concentration and viability of CHO 2F5 in suspension

In Figure 100 the development of product concentration and specific productivity during batch and fed batch cultivation is shown. Except for the value of day 2 the specific productivities in both fermentations are equal. During the first 5 days of both processes they declined considerably reaching about 3 $pg*c^{-1}*d^{-1}$. During one of the batch cultivations done in spinner flasks the specific productivity was kept at 6 $pg*c^{-1}*d^{-1}$ (see Figure 74). As the viable cell integral of the fedbatch fermentation was about 30% larger and the specific productivity of both processes was equal the product concentration at the end of the fedbatch was also increased by 30% and amounted to about 70 mg/l compared to 50 mg/l in the batch process as shown in Figure 100.

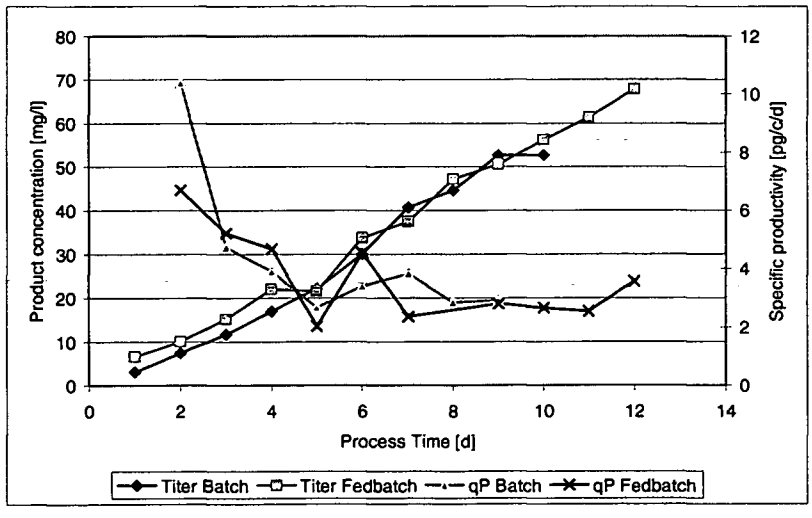


Figure 100: Product concentration and specific productivity of CHO 2F5 in suspension

In Figure 101 and Figure 102 the metabolite concentrations during batch and fedbatch fermentation are shown. In both processes glutamine was depleted on day 6. In the batch fermentation glucose was depleted on day 7 and the maximum lactate concentration was 3 g/l. The glutamate concentration remained at a level of 140 mg/l until day 4. It then started to decline reaching 120 mg/l on day 5 and was finally depleted around day 8 when the con-

centration reached about 30 mg/l. The ammonium concentration increased continually from 40 mg/l on day 2 to 140 mg/l on day 10. The concentration at the end of the batch is substantially higher than the 80 to 90 mg/l that were reached during the two perfusion cultivations.

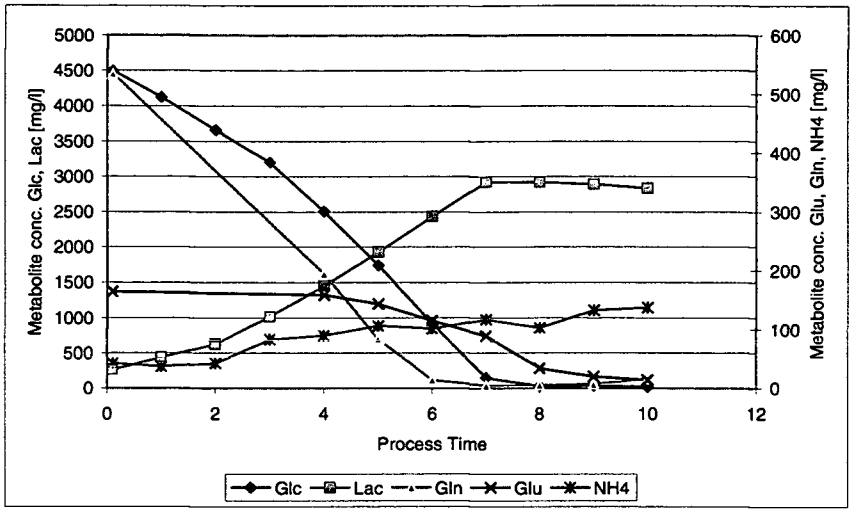


Figure 101: Metabolite concentrations during CHO 2F5 batch cultivation

In the fedbatch fermentation the feed was started on day 4 to keep the residual glucose concentration at 2 g/l. The feed solution contained glucose, aspartate, asparagine, cysteine and glutamate in a 1.5 x medium concentrate as shown in 5.1. During the 8 days from the feed start to the end of the process feed solution equivalent to about 20% of the reactor volume on day 4 was added. As shown in Figure 102 the lactate concentration increased from about 2 g/l at the feed start to 3.7 g/l at the end of the process. The glutamate concentration in the feed solution was higher than required by the cells as it continually increased from 150 mg/l after the feed start to about 350 mg/l at the end of the process. Nonetheless glutamate was obviously used by the cells as the ammonium concentration increases from 70 mg/l on day 6 when glutamine was depleted to 225 mg/l at the end of the process.

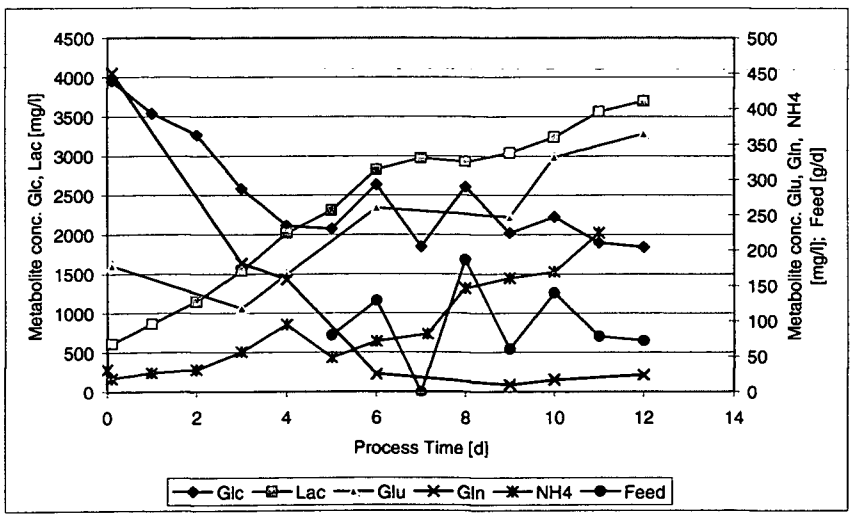


Figure 102: Metabolite concentrations during CHO 2F5 fedbatch cultivation

6.4 Analysis of the lactate dehydrognease concentration in the culture supernatant

The stability of LDH in culture supernatant was tested over a period of 300 days at 4, -20 and -80 °C the results are shown in Figure 103. When stored at –20 °C the activity decreased immediately and was reduced to 65 % after the first day. At 4 °C the enzymatic activity seemed to decrease slightly however when compared to the analytic variability found during the testing of the supernatant stored at –80°C this is not significant. At –80 °C no decline was noted during the 300 days of stability testing. After 300 days the activity was still 92 % of day 1. The coefficient of variation based on 22 measurements during this period was 7%.

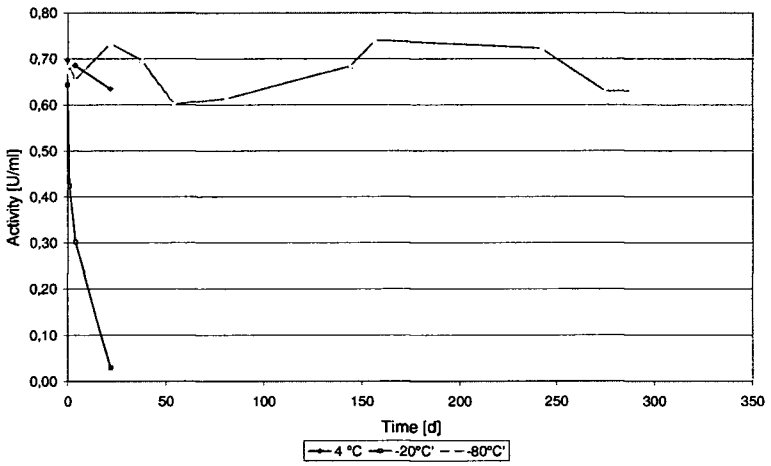


Figure 103: LDH stability at different storage conditions

In Figure 104 and Figure 105 representative pictures of NADH analyses by declining light absorption and fluorescence are shown.

Analysing LDH concentration by following the decrease of light absorption worked well. The linearity of the plot absorption versus time is usually very good as shown in Figure 104. However in this method only one sample can be analysed per run.

Determining the LDH concentration by measuring the fluorescence would allow to analyse several samples at the same time since the fluorescence photometer used in the assay is capable of reading microtiterplates. However there were several difficulties with this analysis. The linearity of the plot fluorescence versus time was usually very good as shown for a representative experiment in Figure 105. Unfortunately the reproducibility of the experimental results was poor. The values for the blanks which showed the decrease in NADH concentration without addition of LDH showed a large difference between various plates. The slopes of the respective lines varied from a decrease of 20 fluorescence units/minute to a decrease of about 200 fluorescence units/minute. Furthermore the enzymatic reaction was faster in the wells at the edge of the plate probably because these wells touched the metal frame of the metering chamber and got warmer due to the better heat transmission. As column 1 and 12 could not be used for analysis the number of samples was reduced. It was tried to reduce the variation of the blank measurements by changing the production lot of the microtiterplate and using fresh reagents. Pipet-

ting errors were excluded by weighing the contents of the individual wells after fluorescence measurement. As the variation of the blank values could not be reduced NADH analysis was done was done by measuring the reduction in light absorption at 340 nm. However due to the capacity limitation NADH concentration was not measured as a routine parameter during fermentations.

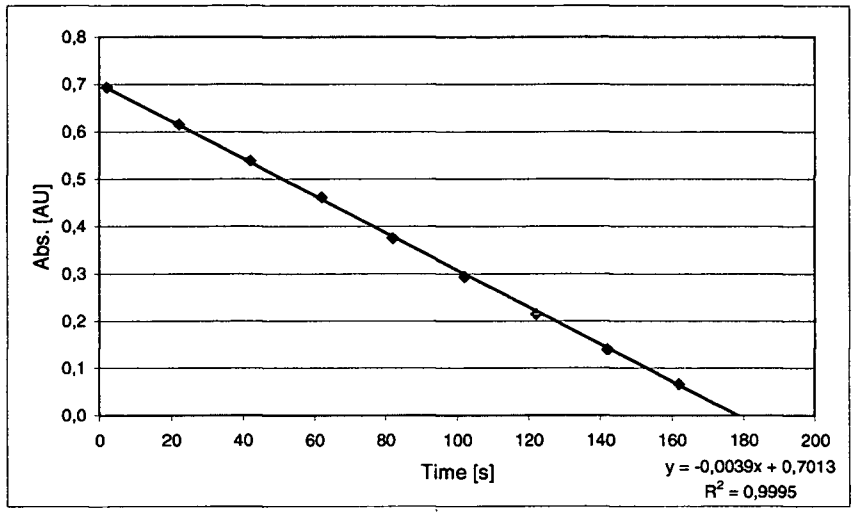


Figure 104: Measurement of NADH concentration by declining light absorption

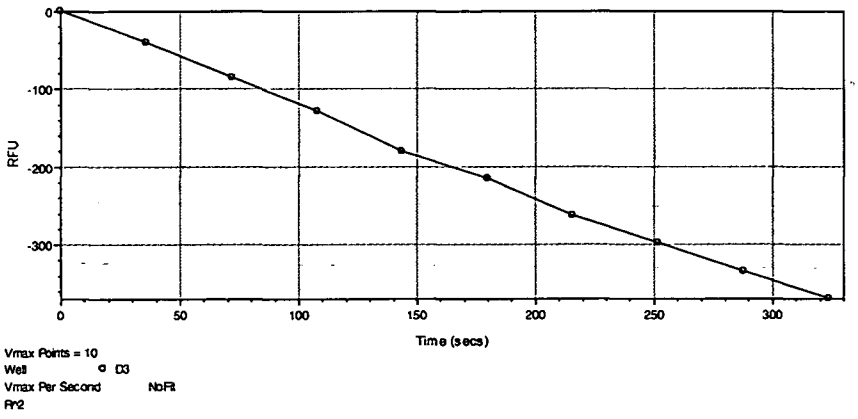


Figure 105: Measurement of NADH concentration by declining fluorescence

To correlate viability determined by hemocytometer count and LDH concentration a batch cultivation of CHO 2F5 cells was done. The results are shown in Figure 106 and Figure 107. The correlation between the two methods is very good as demonstrated by the correlation coefficient of 0.988. As the linear dependence between the viability determined by hemocytometer and the LDH concentration in the supernatant exists during all the 13 days of the batch cultivation there seem to be no problems with LDH stability which correlates well with literature data (56).

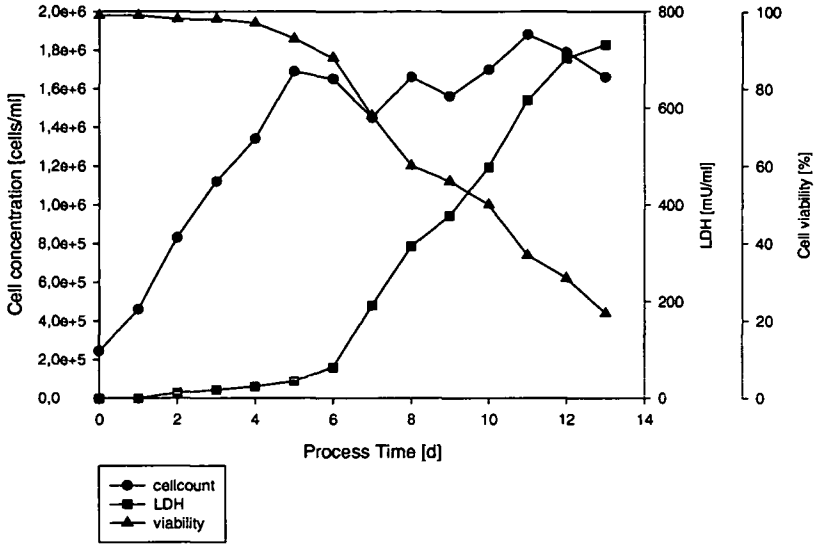


Figure 106: CHO 2F5 batch cultivation – viability and corresponding LDH concentration

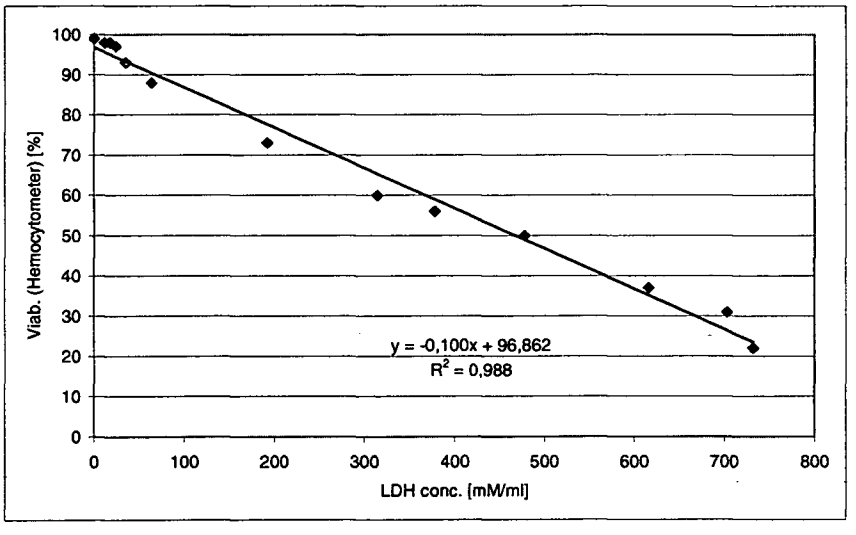


Figure 107: CHO 2F5 batch cultivation – correlation viability and LDH concentration

During a CHO 2F5 fermentation in a FBR samples of culture supernatants were taken at regular interval and analysed for their LDH concentration. In Figure 108 the development of the total cell count and the LDH production per day are shown. The stationary phase of the culture began at day 15, the maximum productivity was recorded on day 13. After day 10 the development of LDH production and total cell count run almost in parallel. On day 23 the cultivation temperature was reduced from 37 to 33°C. This caused a major loss of cells from $5*10^{10}$ down to $3*10^{10}$ cells on day 25. The reduction in the total amount of LDH was even more dramatic as shown in Figure 108. The LDH amount decreased even further from 2000 to 1000 U/d although the cell count remained rather stable between day 25 an 42. When cell count and LDH amount on day 13 and 38 are compared

the viability should be better on day 38. However, this is not reflected in the specific productivity which declined from 2.5 to 1 $pg*c^{-1}*d^{-1}$ as shown in Figure 109. During the first 25 days of the culture the expected relation between specific LDH production which should reflect viability and specific IgG producion is as expected. Until day 15 the LDH amount was low compared to specific productivity this changed on day 15 when productivity declined and the LDH production increased until day 23. After the temperature reduction on this day the specific LDH production and the specific productivity declined in parallel. The declining specific LDH production indicates an increasing viability while the declining specific productivity at rather constant cell number is a hint for suboptimal culture conditions and probably also declining viability.

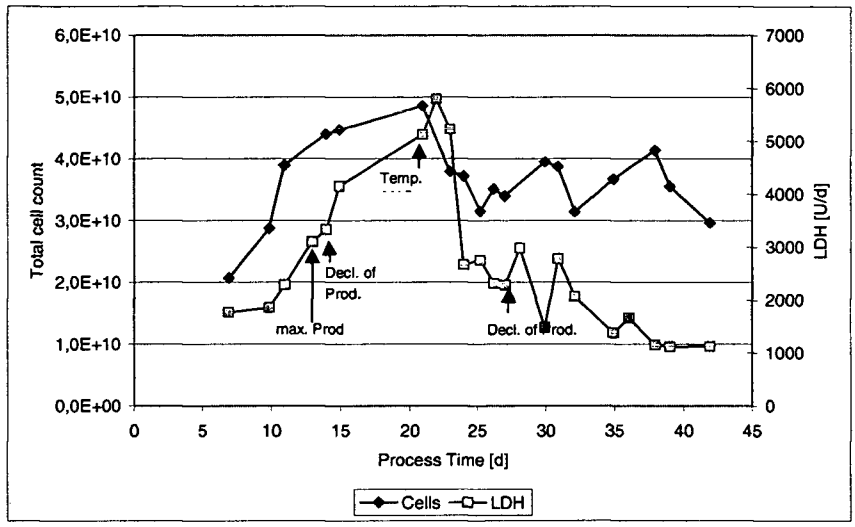


Figure 108: Total cell count and LDH concentration during FBR cultivation of CHO 2F5

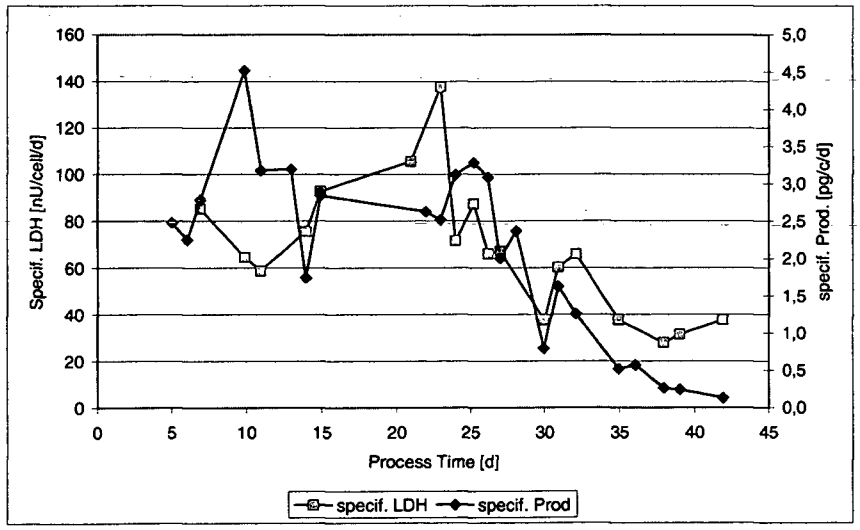


Figure 109: Specific productivity and specific LDH production during FBR cultivation of CHO 2F5

The LDH concentration was also determined at the end of the Cytopore prototype tests shown in Table 17 where CHO 4E10 cells were grown on Cytopore modifications with different charge concentrations. The tests were conducted in spinner flasks run as semicontinuous culture with a daily 50 % media change. At the end of the

cultivation the LDH concentration in the supernatant was measured. The cell count of the cultures varied between 1.9 and $2.6*10^6$ cells/ml. To get an estimation about viability on the different prototypes the LDH concentration per $1*10^6$ cells and ml was compared. In Figure 110 it is shown that the specific LDH concentration varies with the charge density. The optimum cell count was achieved at 0.48 meq/g, the lowest cell concentration of the prototypes depicted in Figure 110 was reached at 1.8 meq/g. The relative LDH amounts for charge concentrations between 0.48 and 1.10 meq/g are in the same range of about 1.125 U/1E+06 cells there is a slight increase in the culture were prototypes with a charge concentration of 1.8 meq/g were used. The Cytopore carriers with a charge concentration of 1.37 meq/g caused an increase in the relative LDH amount of about 13% compared to the culture with a charge concentration of 0.48 meq/g.

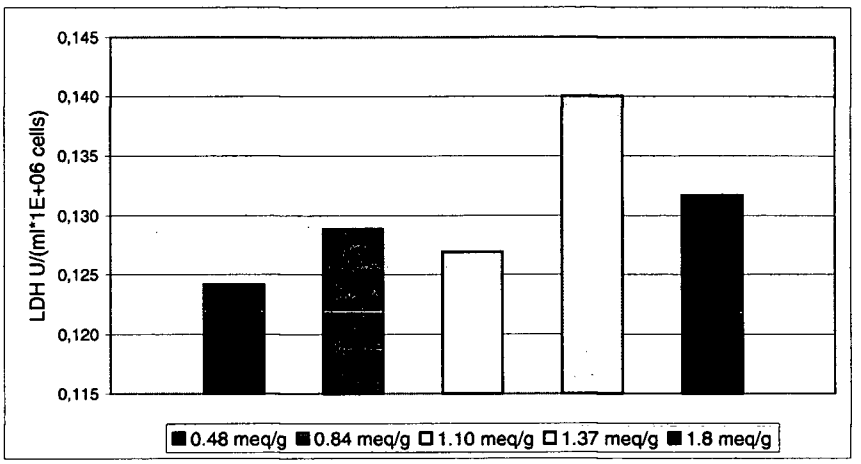


Figure 110: LDH concentration in CHO 4E10 cultures on Cytopore prototypes

To gain further insight in the influence of charge concentration on cell growth and viability of CHO cells on Cytopore carriers the experiment was repeated with a second cell line. CHO 2F5 cells were cultivated on the Cytopore prototypes shown in Table 18. Samples were taken during growth phase at day 4 and at the end of the culture at day 9. Based on the data from suspension culture (see Figure 106) viabilities for cultures on the different Cytopore modifications were calcultated. The results are shown in Figure 111 and Figure 112. As can be seen in Figure 111 there are only minor differences at the beginning of the cultivation although it becomes evident that viability was highest on the Cytopore carriers with a charge concentration of 1.37 meq/g and lowest at a charge concentration of 1.8 meq/g. This situation becomes even clearer when the LDH concentration at the end of cultivation is analysed. As shown in Figure 112 there is a charge optimum at 1.37 meq/g that leads to a 10 % higher viability compared to the cultures on 0.76 and 1.8 meq/g. Although no viability was calculated for the CHO 4E10 cultures shown in Figure 110 the LDH concentrations at the end of cultivation are very similar ranging from 100 to 180 mU/$1*10^6$ cells/ml for CHO 2F5 and from 125 to 140 mU/$1*10^6$ cells/ml for CHO 4E10. Nonetheless the optimum charge concentration for CHO 4E10 cells is obviously lower than for CHO 2F5 cells. While in CHO 4E10 cultures the lowest LDH concentration was found at 0.48 meq/g and the highest at 1.37 meq/g the situation is different for the CHO 2F5 culture. In this case the optimum charge concentration was 1.37 meq/g with a significant reduction in viability at lower and higher charge concentrations. Interestingly, the viability and maximum cell counts do not always correlate. The highest cell counts in the CHO 4E10 cultures were

found at a charge concentration of 0.48 meq/g where also the lowest LDH concentration and highest viability respectively was found. However the cellcounts at 1.10, 1.37 and 1.8 meq/g were almost equal as shown in Figure 40 which is not reflected in the LDH concentration that is significantly higher at 1.37 meq/g.
In case of the CHO 2F5 cultures the maximum cell count was found at 1.1 meq/g and even the cell count at 0.76 meq/g is higher than the one for 1.37 meq/g as can be seen in Figure 43. The specific productivity clearly increased with charge concentration and was highest for 1.8 meq/g.

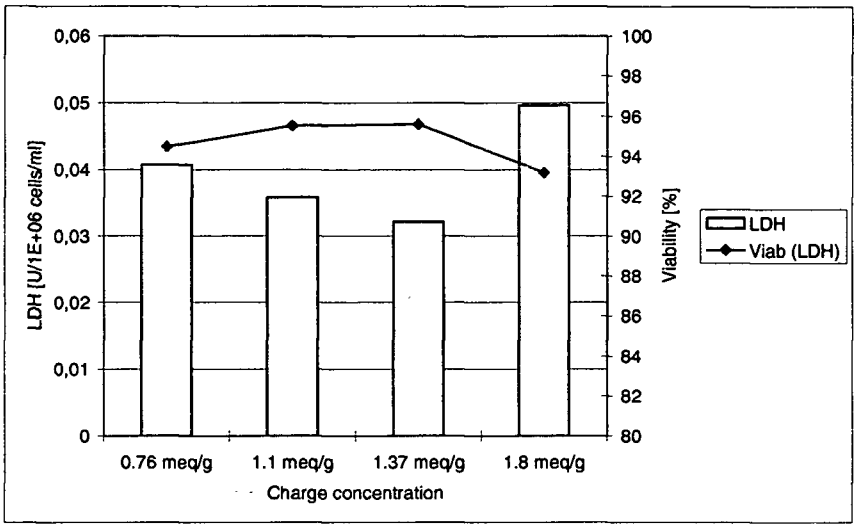


Figure 111: LDH concentration and calculated viability CHO 2F5 on Cytopore on day 4

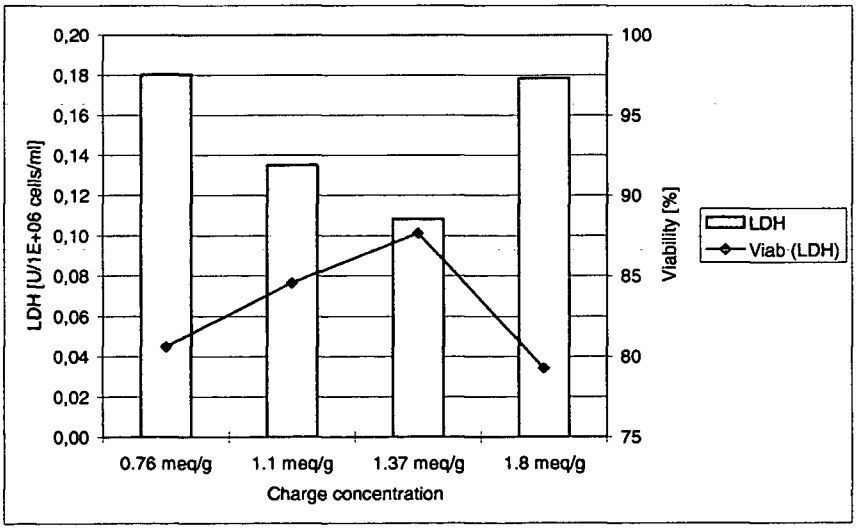


Figure 112: LDH concentration and claculated viability CHO 2F5 on Cytopore on day 9

6.5 3D6 cell line

During the cultivation of different subclones of CHO 4E10 it was noted that some of them showed increased aggregation tendency. Microarray analysis (59) showed that these subclones had a higher expression rate of fibronectin. In suspension culture aggregation is rather unfavourable as cells in the center of the aggregates are subject to oxygen and nutrient limitation. However if the cells are grown on carriers the higher fibronectin expression might lead to increased adherence and be rather advantagous due to reduction of cell loss into the supernatant thus making the process more robust. Furthermore it was planned to analyse changes in specific productivity and product quality between normal cells and cells with elevated fibronectin expression. The adherence characteristics of the C7E5 subclone and the CHO 4E10 parent clone were tested in gelatine coated tissue culture flasks.

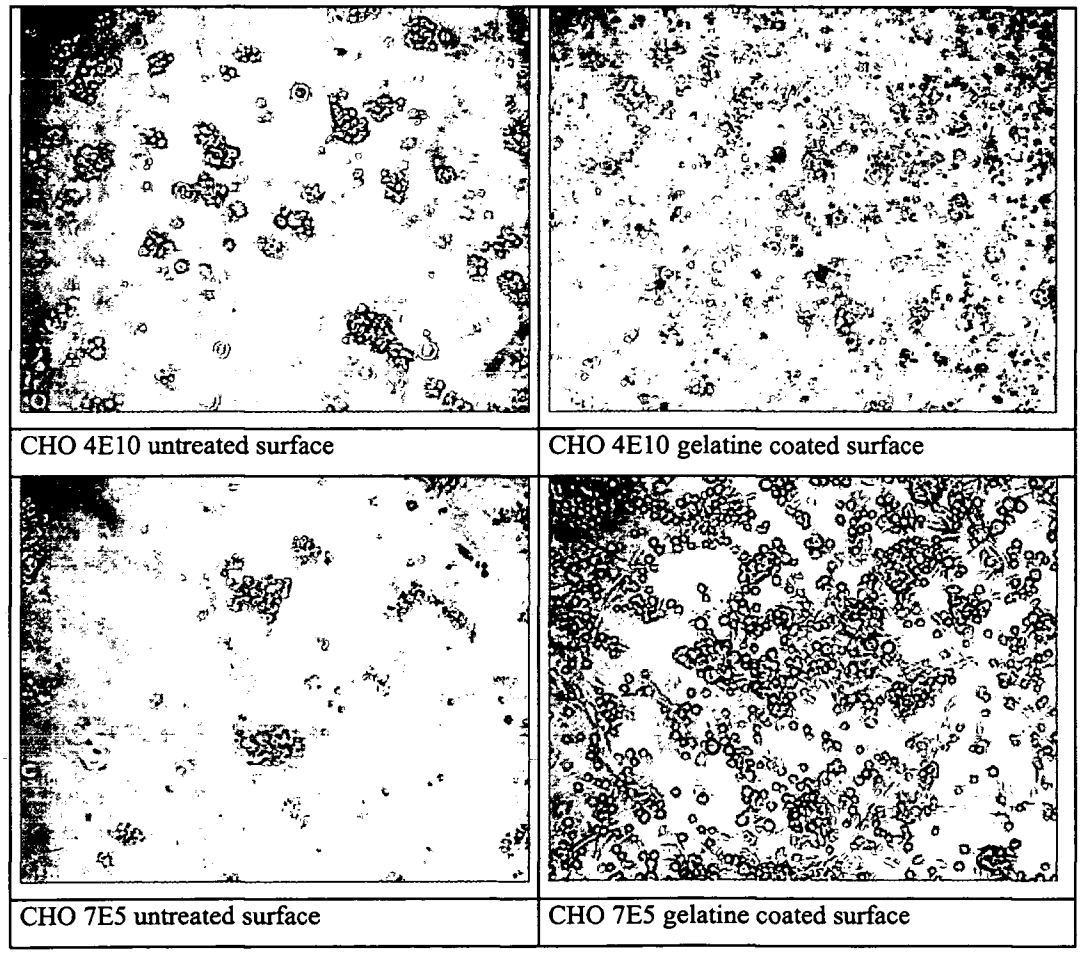


Figure 113: Cell morphology of CHO 4E10 and CHO 7E5 on untreated and gelatine coated surfaces

As shown in Figure 113 the two cell lines show clear differences in their attachment to gelatine coated surfaces. While the major part of CHO 4E10 cells still was in suspension and kept the same rounded morphology that they had on untreated tissue culture flasks a large proportion of the CHO 7E5 cells attached to the gelatinated surface and assumed a flat and longstretched phenotype.

To make use of the increased cell adherence it was tried to establish a cell line expressing IgG and to co-transfect it with fibronectin. The sequence for the antibody was taken from the 3D6 cell line that produces an antibody binding to gp41 of HIV-1. The project was done as diploma work (69).

7 Discussion

7.1 Cytodex Prototypes

About 100 different prototypes were tested regarding their suitabilty for cell cultivation. The prototypes were synthetised by combining 5 different matrices, 11 spacers and 10 types of ligands or surface coatings. Experiments were done in spinner flasks and microtiterplates. The tested microcarriers were inoculated with Vero cells to evaluate cell attachment and cell growth under serum free conditions. Four carriers using arginine as ligand and five that have DEAE as ligand support cell growth equally or better than Cytodex III. The cells also grew well on a prototype that had an RGDS peptide attached to its surface. Additionally microcarriers coated with fish gelatine and recombinant human collagen were tested and gave results comparable to Cytodex III.

Carrier tests in spinner flasks were performed to obtain quantitative data about cell attachment and growth. By relating the results of the prototype cultures to the ones of reference carriers tested in the same run data on the relative performance of the tested carriers was gained and made comparable between different test series.
In a first step the microcarriers were coated with fish gelatine. All three tested prototypes showed a performance that was at least equal to Cytodex III. Although the problematic of viral transmissible spongiform encephalopathy (TSE) should be somewhat alleviated by using collagen from non mammalian origin also fish did not fulfill all demands on the porcine collagen replacement. Next the microcarriers were coated with recombinant human collagen. The first carrier prototype was coated with about 90 mg/collagen per gram carrier matrix. Its performance was practically equal to Cytodex III. Recombinant collagen would of course be a coating of non animal origin however it is much more expensive than the porcine material. It was therefore tried to reduce the amount required to cover the carrier surface. During the coating procedures with the recombinant material there were technical problems to attach the protein to the carrier surface. Cytodex carriers that were to be loaded with 44, 9 and 6 mg collagen/g carrier matrix failed to support cell growth. However, due to the coating problems it is not clear how much collagen was actually attached to the carrier matrix. Two more series of prototypes based on recombinant collagen were directly prepared by Fuji TRL, the manufacturer of the protein. Cells were able to grow on all of them however the maximum cell counts reached only 70 to 90% of the reference carrier Cytodex III.
On the whole the use of fish gelatine or recombinant collagen as coating material resulted in good cell growth but was due to regulatory concerns and tremendous cost increases respectively not considered as appropriate substitution for the porcine collagen coating.
In a further attempt to mimic the surface conditions on collagen coated surfaces an RGDS (arg-gly-asp-ser) peptide was used as ligand and coupled on the carrier surface via an allyl group. Cells attach to collagen coated surfaces via receptor mediated interactions. Integrins are transmembrane proteins that bind to the RGD motif present in many proteins of the intracellular matrix like laminin, vitronectin, collagen or fibronectin (22). It has been shown that RGD containing peptides facilitate cell adhesion to coated surfaces (14). In this publication it was also shown that cell adhesion to the peptide was greatly influenced by the way it was attached to the surface which influenced its accessibility. On the RGDS modified Cytodex carriers a 70 % higher maximum cell number than on Cytodex III was reached however the formation of large carrier aggregates was noticed in the prototype

culture. Although the RGDS peptide showed promising results it was still not a cost effective replacement for the porcine collagen.

As cheap, chemically defined ligands of non animal origin arginine and DEAE were used. Both ligands are positively charged at neutral pH and therefore allow electrostatic interaction between cells and carrier surface. In the case of arginine also cell surface receptors may be involved in the binding to the carrier as the amino acid is part of the RGD motif recognised by integrins.

7.1.1 Arginine carriers

27 Cytodex prototypes modified with arginine were tested in spinner flasks. The experiments provided quantitative data on cell growth. In Figure 114 the relative performance of the tested carriers after 72 h cultivation time is compared to Cytodex III. The period of 72 hours was chosen, as experiments that did not show substantial cell growth (more then 50 % of Cytodex III) were terminated after this time. Four out of the 27 prototypes supported a maximum cell count that was equal or better than Cytodex III. Two of the tested carriers reached a cell concentration between 90 and 110 % of the reference carrier, one reached 113 % and the best one went up to 140 %.

In one series of experiments Sephadex G25 was used as carrier matrix to allow the coupling of increased ligand concentrations however on none of the 5 tested prototypes a cell growth comparable to Cytodex III was achieved although an arginine concentration range from 43 to 100 µmol/ml gel was covered. This was surprising as it had been hoped to increase cell attachment and facilitate cell growth by coupling a higer ligand concentration to the carrier matrix. As also low arginine concentrations that worked on Sephadex G50 did not support cell growth it is likely that the negative results were caused by the matrix material.

Maximum cell concentrations that were comparable or higher than on Cytodex III were only observed on carriers where the arginine was coupled to Sephadex G50 by an allyl group. Although one of the 4 carriers that had BPR butane as spacer supported a cell concentration comparable to Cytodex III after 72 h the maximum cell number reached during the cultivation period was only 80 % of the reference. On the one carrier that was tested with epichlorhydrin as spacer only reduced cell growth was observed. However this negative performance should not be generalised for these spacers as only limited data from 4 and 1 experiment respectively is available.

Cell growth on arginine/allyl modified carriers does not seem to be dependent on the ligand concentration as the two prototypes that supported the highest cell count had a spacer concentration of 129 µmol/ml gel the ligand concentration however was 36 and 12 µmol/ml gel respectively. On the arginine allyl carriers only spacer concentrations between 100 and 150 µmol/ml gel supported cell growth.

As shown in Figure 114 there seems to be a suitable concentration range for arginine/allyl on Sephadex G50. Six out of nine carriers that had at least 50 % of the performance of Cytodex III had a ligand concentration between 35 and 55 µmol/ml gel and a spacer concentration between 110 and 145 µmol/ml gel. However there seems to be an additional factor influencing cell growth besides spacer type and spacer and ligand concentration. There were three arginine/allyl carriers outside the area that also supported cell growth and one carrier insided the mentioned concentration range that did not support cell growth at all. The best evidence for the involvement of an additional factor besides spacer and ligand concentration is given by the comparison of the two carriers U957012 and U1309080. Both have the same spacer/ligand combination. However the former reached a maximum relative cell count of 140 % at a spacer concentration of 129 µmol/ml gel and a ligand concentration of 36 µmol/ml gel while

the latter failed to support cell growth at all despite the specifications being almost equal with a spacer concentration of 125 µmol/ml gel and a ligand concentration of 40 µmol/ml gel.

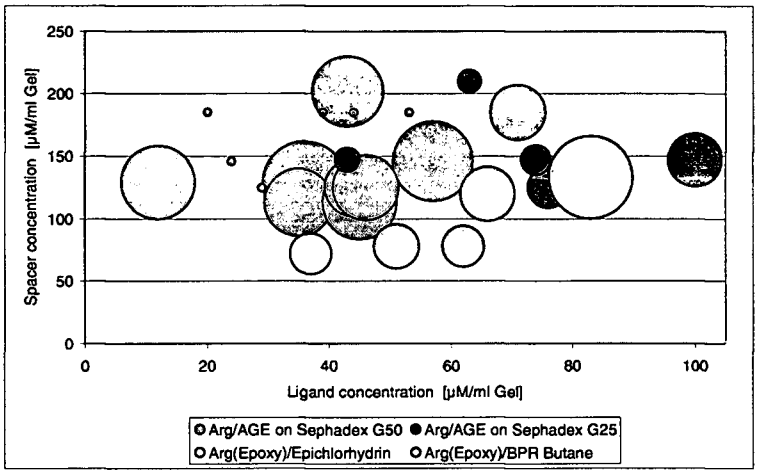


Figure 114: Vero cells were grown on Cytodex arginine prototypes with various spacers and on different carrier matrices. The bubble diameter shows the relative cell growth compared to Cytodex III

During the screenings in microtiterplates further 22 arginine were tested. According to the spacers used for coupling of the ligand they were divided into 7 groups. In Figure 115 the tested ligand/spacer combinations are shown graphically. For Arg/Epoxy and Arg/Epoxy + Allyl Dextran on their respective carrier matricex no spacer concentrations were available therefore these three carrier series are included as bar chart only as shown in Figure 116. For the other seven groups a bubble plot was used to correlate cell growth to spacer and ligand concentration respectively (see Figure 115). As shown none of the tested prototypes supported cell growth. For Arg/Dextran + Allyl and Arg/Mercapto Propionic Acid only a single prototype was tested. Although the result was negative no conclusion can be drawn before a larger concentration range has been tested. Carrier prototypes using arginine coupled by an allyl group to the matrix had already shown positive results in the spinner flask experiments. However in the screening experiments cells did not grow on any of the five tested prototypes. For two of them Sephadex G25 was used as base matrix and they had already been tested negatively in spinner flask experiments. As the negative results for this carrier matrix were reproduced in a static cultivation system this is an additional proof that the failure to support cell growtn was due to the carrier matrix itself and not caused by the increased shear stress as a higher stirring speed was necessary to keep Sephadex G25 carriers in suspension. It is not clear why no cell growth occured on the remaining 3 Arg/Allyl prototypes however one explanation might be the high spacer concentration which ranged between 190 and 320 µmol/ml gel as already in the spinner flask experiments there was only one carrier with a spacer concentration of more than 150 µmol/ml gel that supported cell growth. Arginine coupled via a carboxyl group to the carrier matrix did not support cell growth although ligand concentrations from 15 to 100 µmol/ml gel were tested. Also in this case a very high spacer concentration of 250 µmol/ml gel had been used for the prototypes furthermore the base matrix consisted of Sephadex 6FF which also gave negative results when arginine was coupled to it via an epoxy group or epoxy allyl dextran. A concentration range was only determined for arginine/Dx 40 and arginine/allyl on Sephadex G50 where four and three carriers respectively have been tested as well as for arginine coupled to Sepharose 6FF via

a carboxyl group where four carriers have been tested. For the other spacer/matrix combinations only one or two prototypes were available.

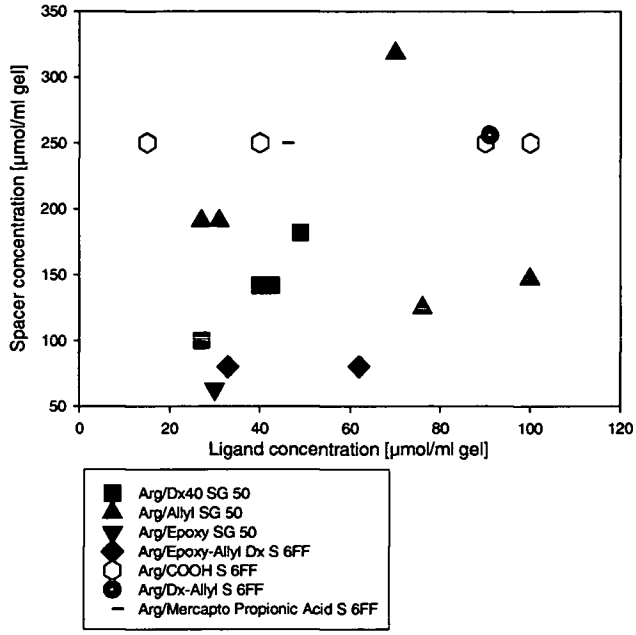


Figure 115: Cell growth on Cytodex arginine prototypes. The plot shows the ligand and spacer concentration for the individual carriers. None of the tested prototypes supported cell growth.

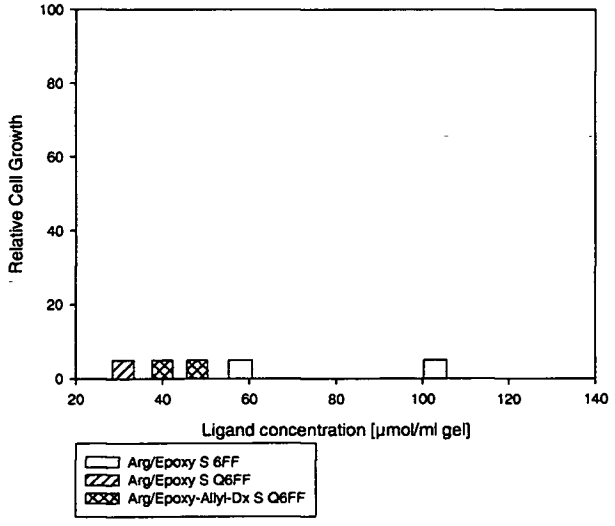


Figure 116: Relative cell growth of Vero cells on Cytodex arginine prototypes with different spacers and carrier matrices

The attachment mechanism of cells to arginine modified surfaces is not fully understood. On the one hand arginine is due to its high pK_a value of 12.5 positively charged at neutral pH. The cells can therefore use charge dependent interaction and the negatively charged cell surface for their attachment (12). However, in this case cell attachment and growth should increase with higher ligand concentrations until an optimum value is reached and

then decrease again. As can be seen in Figure 114 this was not the case for Arginine coupled via an allyl group to Sephadex G50. Good cell growth was observed at a ligand concentration of 12, 36 and 59 µmol/ml gel. The second possible attachment mechanism is the receptor mediated binding of the cells via integrins. As arginine is part of the RGD motif, which is recognised by these cell surface receptors (23). The hypothesis that integrins play a role in cell attachment to arginine modified surfaces is supported by the different behaviour of cells when the ligand is coupled using a different type of spacer. At comparable ligand concentration the surface charge will be the same but the acessibility of the ligand might be altered due to the different steric conditions.

7.1.2 DEAE carriers

In contrast to the 27 arginine carriers only 7 DEAE modified Cytodex prototypes were tested in spinner flask experiments which means that only limited quantitative data about this type of microcarrier is available. On these seven prototypes three different spacers were tested. Due to the small number of experiments the correlation between spacer/ligand concentration and cell growth was only tested for DEAE/Dextran 40k and DEAE/PVA For DEAE/Dextran 500k two different methods were used. First Dx 500k was used as spacer and then DEAE was coupled to it. For the second prototype DEAE was first coupled to Dx 500k and then attached to the carrier matrix. The different coupling methods did not influence cell growth. Analysis data on ligand and spacer concentration is only available for DEAE/Dextran 40k while for the other 2 types of microcarriers only the ligand concentration was determined. As shown in Figure 117 and Figure 118 for DEAE/Dextrane 40k and DEAE/PVA the cell growth seems to be dependent on the ligand concentration as in both cases carriers with higher DEAE concentration showed a better performance. This was also confirmed by experiments in microtiterplates that will be covered in the following section. Interestingly the use of PVA as spacer seems to lower the necessarry DEAE concentration for sufficient cell-carrier interaction. It was almost halved from 75 µmol/ml gel for DEAE/Dx 40 to 40 µmol/ml gel for DEAE/PVA. The tested prototypes with DEAE/Dextran 500k did not support cell growth in the spinner flask experiments. However this results should not be overestimated as only two concentration were tested.

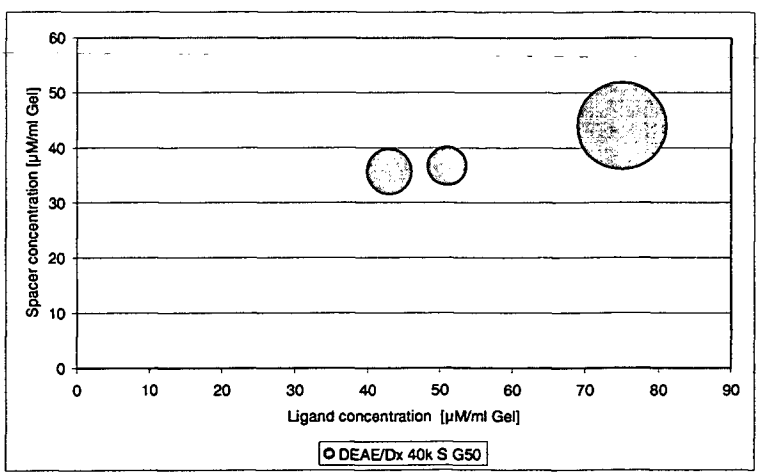


Figure 117: Cell growth on DEAE/Dx 40 modified Cytodex prototypes, the bubble diameter shows the relative cell growth compared to Cytodex III

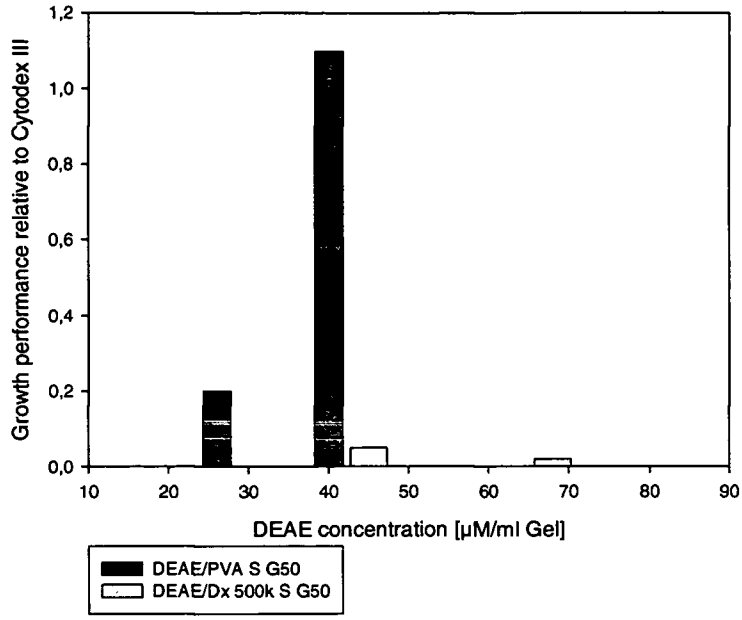


Figure 118: Cell growth on DEAE modified Cytodex prototypes with different spacers

During screening experiments in microtiterplates 17 Cytodex prototypes with DEAE modification were tested, 5 gave positive results. In Figure 119 and Figure 120 the results are shown graphically. As cell growth was evaluated microscopically only the levels good, intermediate and no cell growth are distinguished. To fit the data into diagrams arbitrary numeric levels of 100, 30 and 5 were assigned to the 3 levels.

The evaluation of the results was easiest for prototypes with DEAE as ligand and dextran 40k as spacer as in this case analytical data for ligand and spacer concentration is available. As shown in Figure 119 the optimum ligand concentration for DEAE/Dx40 modified carriers seems to be dependent on the carrier matrix. On Sephadex G50 good cell growth was achieved at ligand concentrations of 63 and 75 µmol/ml gel while at concentrations below 51 and above 110 µmol/ml gel no cell growth occured. This data is in good accordance with the results of spinner flask experiments shown in Figure 117 where cells grew comparable to Cytodex III at a DEAE concentration of 75 µmol/ml gel but cell growth was not supported at ligand concentrations below 51 µmol/ml gel. Furthermore also the DEAE concentration on Cytodex I is about 80 µmol/ml gel. The influence of spacer concentration on the carrier performance can not be assessed from the available data as the concentration varied only between 35 and 42 µmol/ml gel on the 8 prototypes tested in spinner flasks and microtiterplates. In Figure 119 it can also be seen that on Sepharose 4FF a much lower spacer concentration is necessary to couple the same amount of ligand. Either the different base matrix or the low spacer – high ligand combination might facilitate cell growth at almost twice the ligand concentration compared to Sephadex G50. However as only one prototype was tested the range of suitable ligand concentration on Sepharose 4FF remains to be defined.

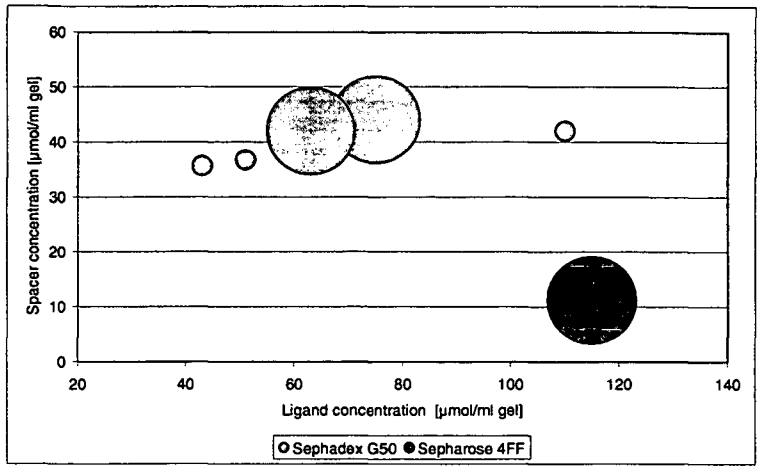


Figure 119: Cell growth on DEAE/Dx40 modified carriers with different matrices

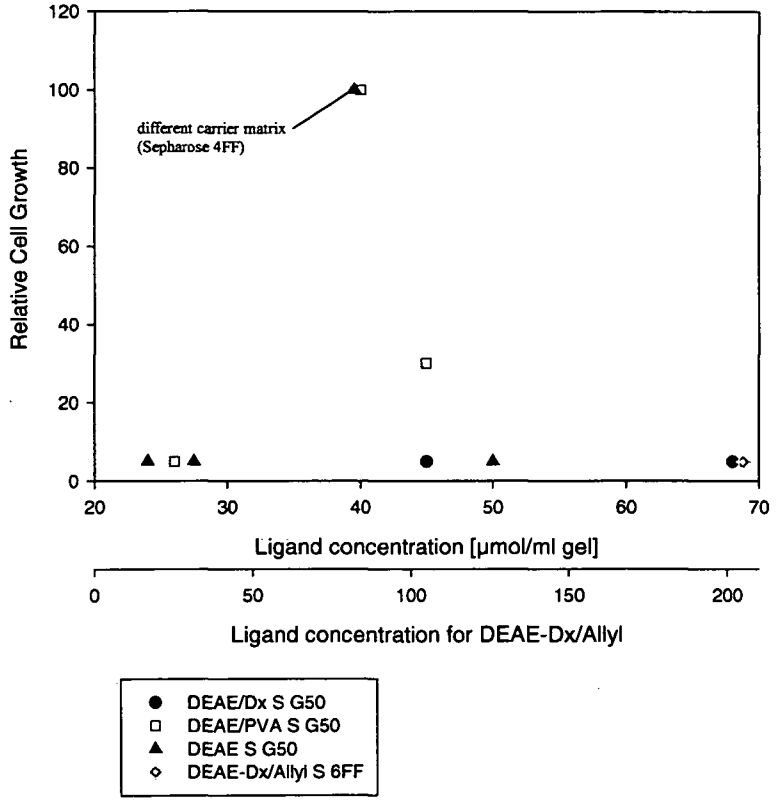


Figure 120: Cell growth on DEAE modified carriers with different spacers

In Figure 120 the relative cell growth on DEAE modified Cytodex prototypes with different spacers is shown. There are two carriers that clearly show a positve performance. Cell growth on DEAE/PVA prototypes seems to be concentration dependent. There was no cell growth at a ligand concentration of 26 µmol/ml gel, when the ligand concentration was increased to 40 µmol/ml gel the cells grew well and at a still higher ligand concentration of 45 µmol/ml gel the cell growth was reduced again. Also in this case the results of the static system were confirmed by spinner flask experiments as shown in Figure 118. DEAE/PVA prototypes with a DEAE concentration of 26 µmol/ml gel did not support cell growth and reached only 20 % of the cell concentration of

Cytodex III after 3 days while at a DEAE concentration of 40 µmol/ml gel a maximum cell count of 110 % of Cytodex III was reached. Vero cells did not grow on the tested DEAE prototypes where dextrane was used as spacer and on the one prototype that had DEAE-Dx as ligand and allyl as spacer. However as only two and one concentration respectively were tested no final conclusion can be drawn. Cytodex carriers that had DEAE directly attached to the matrix gave further evidence that the ligand concentration has to be seen in connection with the spacer used and the matrix it is attached to. On carriers where DEAE was attached to Sephadex G50 no cell growth occured at a ligand concentration of 24, 28 and 50 µmol/ml gel. On Cytodex I carriers which are also based on Sephadex G50 a DEAE concentration of about 80 µmol/ml gel is used so the concentrations used on the prototypes might be to low to allow sufficient interaction between carrier and cells. However when Sepharose 4FF was used as matrix vero cells grew on the carriers at a ligand concentration of 40 µmol/ml gel. Also the use of PVA as spacer obviously reduces the necessary ligand concentration as cells grew well at a DEAE concentration of 40 µmol/ml gel.

In case of DEAE as ligand the charge dependent interaction of the cells with the modified surface is obvious. An optimum ligand and thus also charge concentration was shown for DEAE coupled directly to the matrix as well as for DEAE coupled to the matrix via PVA. However in spite of cell attachment being charge dependent there also the matrix seems to influence cell attachment. When Sepharose 4FF was used the cells grew at DEAE concentrations of 40 and 115 µmol/ml gel. Using Sephadex G50 these two concentrations were either too low or too high. Thus the acceptable concentration of the ligand seems to be influenced by the matrix used. The mechanism for this phenomenon is not clear as there is not only a shift of the optimum ligand concentration but even a broader concentration range. Nonetheless this could be used to develop a carrier with lower charge concentration and thereby facilitate cell detachment during upscale.
Alltogether 7 DEAE prototypes gave results comparable to Cytodex III. However as the attachment mechanism to these carriers is different from the one to collagen further experiments with cell lines requireing a collagen coated surface will be necessarry to show if they can replace Cytodex III.

7.1.3 Carriers with other ligands

On eight Cytodex prototypes other ligands were immobilised to either Sephadex G50 or Sepharose 6FF. The results are shown graphically in Figure 121. The ligands were lysine, agmatine (the decarboxylation product of arginine), arginine substituted with an methyl or ethyl group and finally Fmoc (9-fluorenylmethyl carbamate) protected arginine. The ligands were linked to the matrices either by an allyl group, a carboxyl group or an amino group. For amino coupled Fmoc-arginine 2 different ligand concentrations 90 and 127 µmol/ml gel were tested on a third prototype the ligand concentration was not determined. The other matrix-spacer-ligand combinations were tested with only one prototype each. Some cell growth occurred on methylated arginine linked to Sephadex G50 via an allyl group. Vero cells did not grow on the other seven prototypes however as only one or two concentrations of each ligand were tested no general conclusion on their suitability can be drawn.

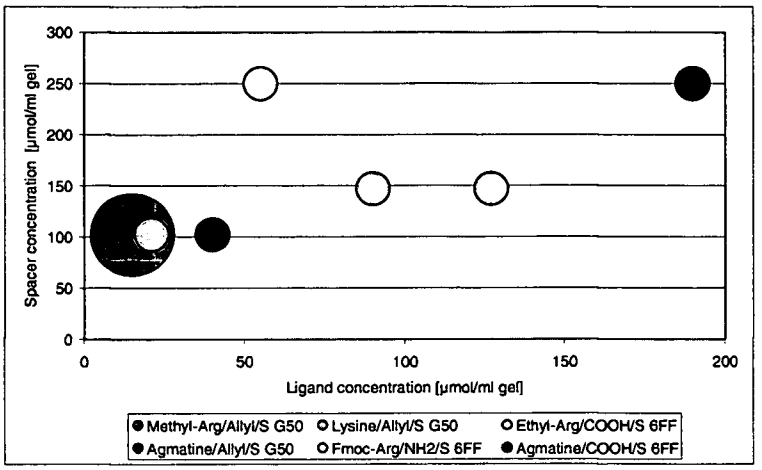


Figure 121: Cell growth on Cytodex prototypes with various spacer and ligand combinations

As the arginine itself also the functional group of its derivates methyl-arginine, ethyl-arginine, Fmoc-arginine and agmatine is protonated at physiological pH. Lysine has a lower pK_a value than arginine but is still positively charged at neutral pH. The attachment mechanism to the arginine derivatives is probably the same as to arginine and therefore not purely charge dependent. As shown in Figure 121 an increasing ligand concentration did not have a positive effect on cell attachment. If on the other hand the interaction between cells and carriers does at least involve integrins this might explain why the cells attached to methyl-arginine but not to lysine at the same ligand concentration. As the RGDS peptide showed promising results it might be useful to test dipeptides as ligands. This would possibly strengthen the receptor interaction with the carrier surface and pose an economically viable compromise to the expensive RGDS.

The GCSS showed promising results when tested with Cytodex III carriers. The system allows the non invasive measurement of cell growth in 96 well plates by registering the light absorption. However due to the incomplete coating when other carriers than Cytodex III were used the background signal due to cells growing directly on the plate material was too high which made the GCSS unsuitable for carrier screening. In a preliminary experiment modified dextrane was successfully coated on microtiterplates and cell growth on these coatings seemed to be influenced in a concentration dependent manner. The dextrane formed a homogenous, transparent coating in the microtiterplates. If the coating protocoll can successfully be transferred from 12 well plates to 96 well plates also further experiments in the GCSS might be considered.

7.2 Cytopore Microcarriers

The cultivation of CHO cell lines on Cytopore carriers with different charge concentration showed that the cells need a minimal charge for attachment to a surface. As shown in Figure 122 practically no cell growth occurs below a charge concentration of 0.5 meq/g. At this point the cell/carrier interaction is sufficient for CHO 4E10. It also seems to be the optimum charge for this cell line as the maximum cell numbers obtained during 10 days of cultivation get lower with increasing charge concentration. For the second tested cell line CHO 2F5 the situation is similar although a shift of the optimum charge concentration was observed. As can be seen in Figure 122 this cell line needs a charge concentration of more than 0.4 meq/g for cell growth on the carriers. At 0.76 meq/g a sudden increase in cell number is visible, the maximum cell number is then reached at a charge concentration of 1.1 meq/g while at higher charges a considerable reduction of the maximum cell number can be seen. The requirement of a minimum charge for cell/carrier interaction is also reflected in the kinetics of cell attachment. Both cell lines showed practically no attachment to the lowly charged prototypes during the inoculation period of 6 hours. With increasing charge concentration (0.48 meq/g for CHO 4E10 and 0.76 meq/g for CHO 2F5) more than 90 % of the cells attached to the carriers within one hour. Due to speculation about a cytotoxic impact of surface charge on CHO cells (personal communication with EMD Chemicals Inc.) the viability of the cells in the supernatant was analysed by trypan blue exclusion. Independent of the prototype's charge concentration the viability was better than 95 % throughout the 6 h inoculation period and no decline was noticed during this time.

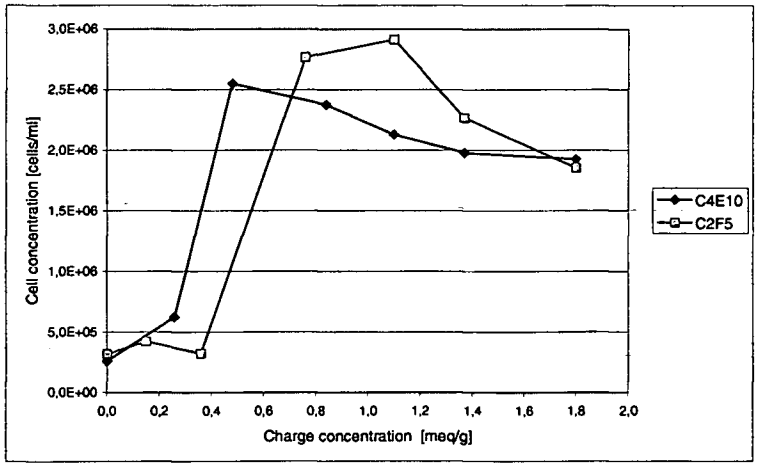


Figure 122: Two IgG expressing CHO cell lines were cultivated on Cytopore prototypes with different charge concentrations. The graph shows the maximum cell counts reached on the various prototypes.

It should be pointed out that the optimum charge for cell growth is not necessarily also the suitable environment for maximum antibody production. For CHO 2F5 the mean specific productivity was about 30% higher on Cytopore prototypes with 1.8 meq/g compared to 1.1 meq/g where optimal growth occurred. Additionally also the decrease in specific productivity was much lower for cells grown on carriers with higher charge concentration. The experiments were done in spinner flasks which means that control of the environmental conditions was limited to daily medium change however due to the extremely high cell numbers pH control was obviously

problematic. Additionally the 50% medium exchange was at least in the case of the culture on carriers with 1.8 meq/g on the last day not sufficient to prevent nutrient limitation as the residual glucose level fell to about 200 mg/l while it remained at 500 mg/l in the other cultures. Also the cell concentration in the supernatant increased earlier in the cultures on Cytopore prototypes with 0.76 and 1.1 meq/g (data not shown). The cell detachment occured most probably not due to space limitation as during bioreactor cultivation more than $1.2*10^7$ cells/ml were accomodated using a carrier concentration of 2 g/l compared to the 1 g/l used in the spinner flasks. It is therefore more likely that the deterioration of the environmental conditions especially the low pH value caused the increase of the cell concentration in the supernatant. The higher charge concentrations of 1.37 and 1.8 meq/g allow a stronger cell/carrier interaction and seem to make the process more robust. In the spinner flask the total productivites showed rather small variations and were only 14 % higher on Cytopore with 1.8 meq/g compared to 0.76 meq/g as the higher cell numbers partly made up for the lower specific productivity. The antibody concentration achieved in a reference batch culture with suspension cells was about 2.5 times higher than in the semicontinuous perfusions however the average volumetric productivity was 60 to 90% increased in the perfusion cultures. Furthermore there has to be taken into account that a daily 50 % medium change was necessarry in the perfusion cultures to obtain a minimum of pH control therefore in both systems the same medium was used and no effort was made to increase glucose/glutamate levels to achieve higher product concentrations due to reduced perfusion rates. During a long term perfusion process in a bioreactor the robustness of the process on the more highly charged carriers is likely to be beneficial as the higher specific productivity will significantly increase the total yield and additionally a lower cell concentration in the supernatant reduces the problem of biofouling.

7.3 Bioreactor cultivations

Four antibody producing CHO cell lines were tested on Cytopore I and Cytoline I carriers in perfusion processes. CHO 2G12, CHO 2F5 and CHO 4E10 produce neutralising IgG antibodies directed against HIV the fourth cell line CHO HB617 produces an IgM antibody against the GM1 ganglioside. The purpose of the experiments was to compare antibody production in a classical batch and fedbatch process to production in a perfusion process with the cells immobilised on microcarriers. The inoculum for the microcarrier based processes was generated either in spinner flasks or in a bioreactor with the CHO cells growing in suspension. It soon became clear that due to the inoculum preparation the use of a robust cell line which is insensitive to shear stress is a prerequisite for successful fermentation even when the cells are eventually cultivated on macroporous carriers that offer good protection against shear stress. This was especially evident with the two cell lines CHO HB617 and CHO 4E10 that had to be adapted to the shear stress in a stirred tank reactor by repeated batch cultivation over several weeks until viability and cell number at the end of the batch reached satisfying levels and allowed the start of the microcarrier based cultivation process.

The four tested cell lines did not show a uniform picture concerning their performance when cultivated on microcarriers. The differences were especially distinct on Cytoline I carriers.

CHO 2G12 was the only cell line where a substantial decrease of the specific productivity occurred on both carrier types. On Cytopore carriers it decreased from about 12 to 5 $pg*c^{-1}*d^{-1}$ over the course of two weeks which is a significant reduction compared to the 8-10 $pg*c^{-1}*d^{-1}$ that were reached in suspension culture. The same development was also observed on Cytoline carriers. In this cultivation system the specific productivity declined from about 15 to less than 5 $pg*c^{-1}*d^{-1}$ within the first two weeks and then stabilised in the range of 4 $pg*c^{-1}*d^{-1}$ during the following two weeks until the end of the process.

The performance of the CHO HB617 cell line in the carrier based cultivation systems was completely different. During the growth on Cytopore I carriers the specific productivity dramatically increased and finally with about 60 $pg*c^{-1}*d^{-1}$ reached the threefold level compared to the 20 $pg*c^{-1}*d^{-1}$ obtained in suspension culture. The higher specific productivity in combination with the increased cell concentration led to an almost 6 fold increase in volumetric productivity from 12 to 70 $mg*l^{-1}*d^{-1}$ and a doubling of product concentration from 60 to more than 140 mg/l when the perfusion culture on Cytopore I carriers is compared to repeated batch culture in suspension. It is especially noteworthy that the doubling of the product concentration was achieved although the same medium was used for batch and perfusion cultivation as normally in perfusion processes an enriched medium is used to decrease the perfusion rate and thereby increase the product concentration (32, 36). On Cytoline I carriers the specific productivity of CHO HB617 cells reamained constant at about 25 $pg*c^{-1}*d^{-1}$ during the cultivation period of 30 days. This cell line was therefore the only one that reached the same specific productivity in fluidised bed and suspension cultivation.

CHO 4E10 cells showed the same specific productivity of around 8 $pg*c^{-1}*d^{-1}$ during 50 days of suspension cultivation and subsequent more than 40 days of perfusion culture on Cytopore I. Due to the fivefold increase in cell concentration of up to $4.5*10^6$ cells/ml in perfusion culture also the volumetric productivity rose from about 5 to more than 20 $mg*l^{-1}*d^{-1}$. The product concentration was more than doubled from about 20 to 50 mg/l. On Cytoline I carriers the cell line had a substantially reduced specific productivity of about 3 $pg*c^{-1}*d^{-1}$ during the

initial phase of the culture. After temperature reduction to 34°C the specific productivity increased to around 4 $pg*c^{-1}*d^{-1}$.

The CHO 2F5 cell line, like the CHO 4E10 line clearly preferred the cultivation on Cytopore I carriers to Cytoline I carriers. During the first cultivation on Cytopore I carriers the specific productivity remained constantly at 6 $pg*c^{-1}*d^{-1}$ and hence at the same level as in suspension culture. The cell concentration during cultivation on Cytopore I was increased fivefold compared to growth in repeated batch culture and reached a maximum value of $1.3*10^7$ cells/ml. Compared to batch cultivation the volumetric productivity in perfusion culture was increased about eightfold to more than 40 $mg*l^{-1}*d^{-1}$. During the initial phase of perfusion culture the product concentration rose only slightly but after lowering the residual glucose concentration and thereby also the necessarry perfusion rate it was increased about fivefold to more than 100 mg/l. In contrast to this during cultivation on Cytoline I carriers there was a dramatic decrease in specific productivity during the initial phase of the culture. In all three cultivations of CHO 2F5 cells on Cytoline I a decline of specific productivity from about 10 to around 2 $pg*c^{-1}*d^{-1}$ during the first seven days was observed. After a reduction of the perfusion rate in the last experiment the specific productivity increased again to about 4 $pg*c^{-1}*d^{-1}$ however this is still only half the initial value and only about 60 % of the level reached in suspension culture and on Cytopore I. For the CHO 2F5 cell line by far the most process optimisation has been done. The medium was adapted for the perfusion process by increasing glucose and amino acid concentration according to spent media analysis and the aeration during perfusion cultivation on Cytopore I was optimised. As shown in Figure 123 the average volumetric productivity in perfusion processes was six to eightfold increased compared to the batch and fedbatch process respectively. Due to the medium optimisation and hence the reduced perfusion rate the product concentration in the Cytoline I based fluidised bed cultivation reached the same level as in the fedbatch process (70 mg/l) and was even increased by 30 % to 90 mg/l in the Cytopore I based perfusion process.

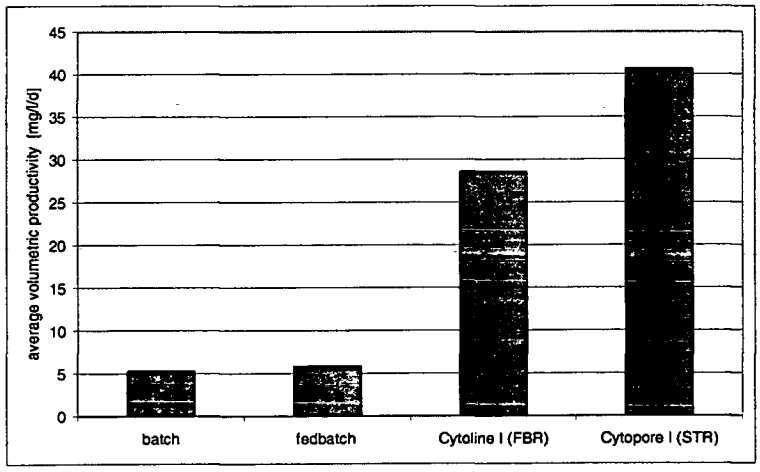


Figure 123: Average volumetric productivities of CHO 2F5 cells in different cultivation systems

All in all the volumetric productivity in perfusion cultures is increased 6 to 8 fold compared to batch cultivation. The Cytopore I processes seem to be more robust compared to cultivation on Cytoline I carriers. The cause of the dramatic decline in specific productivity during the cultivation of CHO 2G12 and CHO 2F5 cells on Cytoline I carriers remains to be determined. It already happens during the first few days of the process when the nutrient levels are still high and a limitation concerning residual glucose or amino acids can be excluded. Furthermore the

cell concentration during this phase does not exceed 5 to $6*10^7$ cells/ml carrier which means that less than half of the available space on the carriers is populated. Therefore also oxygen limitation which has been reported to be problematic in fluidised bed fermentations (58) is rather unlikely especially since CHO 4E10 and CHO HB617 cells have been cultivated at comparable cell concentrations for 25 to 30 days without reduction of the specific productivity. Taking into consideration the increase of the specific productivity in a CHO 2F5 cultivation when the perfusion rate was reduced from 18 to 3 carrier volumes per day the decline might be caused by the inability of the cells to condition their environment when the perfusion rate is too high.

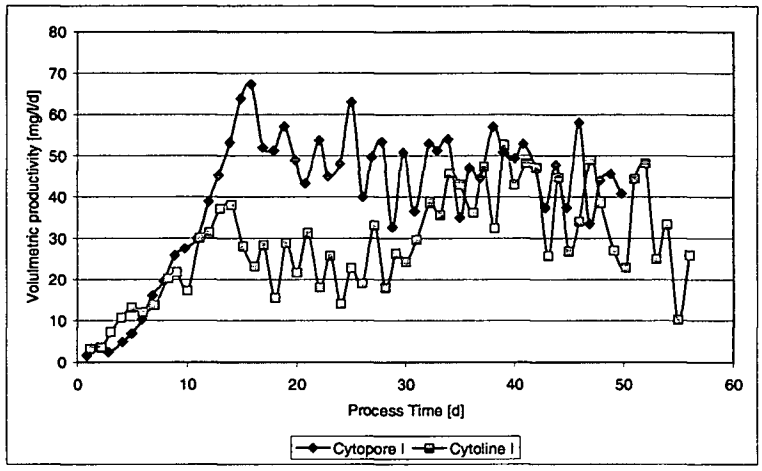


Figure 124: Volumetric productivity of CHO 2F5 cells on Cytopore I and Cytoline I carriers

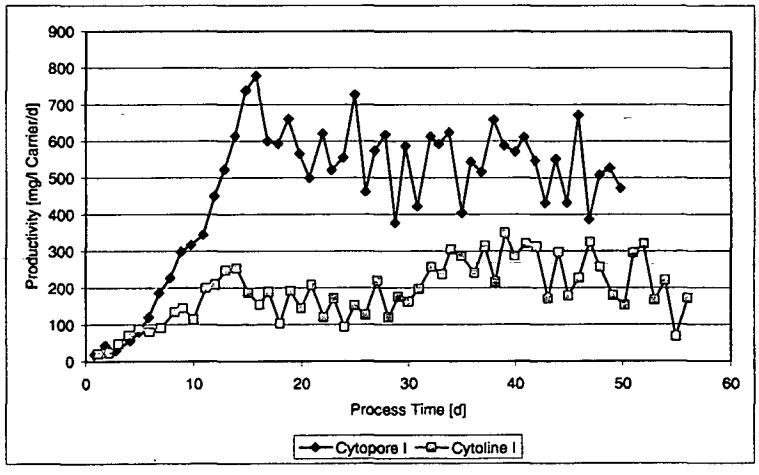


Figure 125: Productivity of CHO 2F5 cells per l carrier shown for Cytopore I and Cytoline I

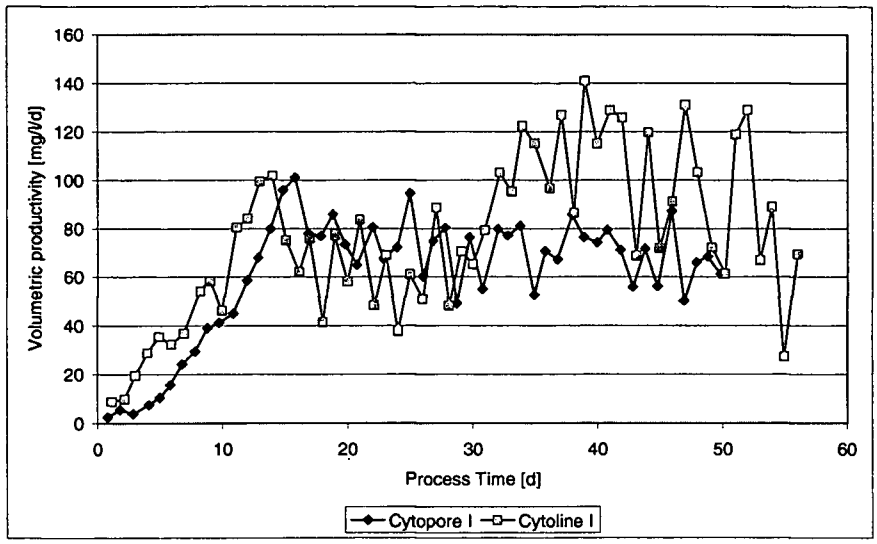


Figure 126: Extrapolated volumetric productivity for CHO 2F5 cells at a Cytopore I concentration of 3 g/l and a Cytoline I concentration of 40 % of the reactor volume

In the STR and the FBR that were used for the cultivation of CHO 2F5 the ratio of carrier volume to reactor volume were quite different. Cytopore I carriers made up about 8 % of the reactor volume in the STR while the Cytoline I carriers made up 15 % of the volume in the FBR. Despite having about the same cell concentration per l carrier (data not shown) the volumetric productivity in the FBR is generally lower than in the STR as shown in Figure 124. This is due to the reduced specific productivity of CHO 2F5 cells on Cytoline I carriers. As can be seen in Figure 125 this cell line reaches at maximum half the productivity per l carrier on Cytoline I compared to Cytopore I. Due to technical constraints to make the use of standard pH and pO2 probes possible in the Cytopilot Mini the Cytoline I carriers make up only 15 % of the reactor volume. In large scale the carrier compartment would be relatively bigger and the carriers would make up 40 % of the reactor volume. For the Cytopore I carriers the maximum concentration in a stirred tank reactor would probably be 3 g/l corresponding to about 12 % of the reactor volume. The limiting factor in the latter case is to provide enough oxygen without excessive foaming. It is easier to increase carrier concentration and thereby also cell concentration in the FBR as sparging can be used for oxygen supply because Cytoline I carriers have a 30 % higher density than Cytopore I carriers and are therefore not transported to the liquid surface by attached air bubbles. In Figure 126 it is shown that in case of the increased carrier concentrations the volumetric productivity in an FBR would be equal or even superior to the STR despite the significant reduction in specific productivity.

7.3.1 Development of a retention device for Cytopore I carriers

In this work 3 different retention techniques for Cytopore I carriers were tested. The first was a static retention sieve located inside the reactor (6 l wv) with a mesh size of 100 µm and a surface area of about 50 cm². During the first perfusion cultivation which lasted 17 days no problems were observed. However with increasing process duration biofouling became problematic and the sieve could not be cleaned by backflushing.

As a second possibility carrier retention via a hollow fiber cartridge was tried. The cartridge was part of an external loop and culture supernatant was removed by crossflow filtration. During the cultivation of CHO HB617 on Cytopore I carriers the retention was operated for more than 2 weeks without reduction of the permeate flow and seemed insusceptible to biofouling. A critical parameter however was the shear stress that was inflicted on the cells by the continuous cross flow through the hollow fibers. While a crossflow rate of 90 reactor volumes per day led to cell detachment the reduced rate of about 25 reactor volumes per day was well tolerated. In contrast to this during the use in a CHO 4E10 culture on Cytopore I a hollow fiber cartridge used as carrier retention was blocked after just 2 days. This was especially surprising as a cartridge with a larger pore size of 0.45 µm instead of the former 0.2 µm had been used that should be even less prone to biofouling. In general it seems possible to use hollow fiber cartridges for carrier retention however preliminary tests are necessary to find the right pore size and determine the maximum tolerable shear stress inflicted by the crossflow through the module. The selection of the pump influences the created shear stress. At laboratory scale normally peristaltic pumps are used in this case it is important to select a pump tubing with large diameter to keep the pump speed as low as possible. The maximum allowable crossflow is the most important limitation of the hollowfiber system since it has to be increased in accordance with the permeate flow to avoid fouling of the membrane. However, the technique is scaleable and its use for cell retention has been described in literature (47).

The third tested retention technique was the use of gravity settlers. After testing of numerous devices the apparatus shown in *6.3.1* was developed. During preliminary tests it was shown to retain Cytopore I carriers up to flow rates of 17 l/d while it has only an internal volume of about 150 ml. The performance is however dependent on a laminar flow that is used to create a suction on the lee side. As the formation of such a flow is generally hindered by the fixtures and tubings in a bioreactor that create turbulence it has only been successfully operated at perfusion rates of up to 9 l/d during fermentation. Nonetheless it was sufficient as the bioreactor was usually operated at a working volume of 6 l. In large scale the problem of turbulence formation by fixtures would be alleviated as their impact on fluid dynamics decreases in a larger reactor. The settler proved to be a simple and robust retention device. No problems with biofouling were observed although it has been used in perfusion processes with a duration of 50 days. In literature gravity settlers have mainly been described as devices to retain suspension cells(51, 52). In this context it has been stated that their scale up is limited due to the required internal volume and so far only perfusion rates of up to 100 l/d have been reported. However the use in microcarrier culture dramatically reduces the required internal volume of the settler as the cells are immobilised on carriers that have a higher density than mammalian cells and therefore the settling time is reduced. Consequently the residence time of the cells in the rather uncontrolled environment of the settler is shortened.

7.3.2 Optimising the cultivation medium

For the first perfusion cultivations the same medium as for the batch cultivations was used. As these media had a relatively low glucose concentration high perfusion rates were necessary to maintain the desired residual glucose level. The high perfusion rates in turn led to low product concentrations and sometimes also exceeded the capacity limits of the carrier retentions. To lower the perfusion rates first the glucose concentration of the medium was increased. However doing so led to an unbalanced medium concerning the ratio of glucose and amino acids. By spent medium analysis the limiting amino acids were defined and their concentration increased. For the CHO 2F5 cultivation the glucose concentration was increased from 3.1 up to 5.5 g/l and the amino acids then raised to

the levels described in 5.1. In combination with a lower residual glucose concentration the perfusion rates were reduced to 20 – 30 % of the former maximum values which led to an up to sixfold increase in product concentration. An interesting aspect of perfusion culture was that the ammonium levels were 50 to 60 % lower than during batch cultivation although the same glutamine concentrations had been used for both processes.The perfusion cultivation allows to tightly control the residual glucose concentration. It is known that the specific consumption rates of nutrients generally decline with the available concentration of the respective nutrient. Maintaining key nutrients at low levels is thus a usfule strategy to reduce the formation of undesired metabolites like ammonium or lactate (33, 46). Also in the CHO 2F5 fermentations the specific glucose consumption of the cells decreased when the residual glucose concentration was reduced below 1 g/l. Additionally also the specific lactate production of the cells decreased probably due to the elevated lactate level. The combination of these two factors resulted in a 20 % reduction of the glucose/lactate conversion rate which means that the energy metabolism of the cells got more efficient. For CHO 2F5 cells it was observed that the most significant reduction in glucose consumption was achieved by reducing the residual glucose concentration from 1 g/l to 0.5 g/l. Below a glucose level of 0.3 g/l cell detachment from Cytoline carriers was noticed. The lactate concentration increased when the cells were provided with a higher glucose concentration. The maximum tolerable lactate concentration for CHO 2F5 cells was found to be about 2.5 to 3 g/l on Cytopore I carriers and up to 3.5 g/l on Cytoline I carriers. At higher lactate concentrations cell detachment from the carriers occurred. A reentry of lactate into the cells at high concentrations exceeding 4 g/l has been reported in literature (60) but for CHO 2F5 cells was neither noticed in perfusion nor in fedbatch cultivations. To achieve a low perfusion rate and at the same time prevent cell detachment either due to low residual glucose concentration or excessive lactate concentration an initial glucose concentration of 5 g/l and a residual glucose concentration of 0.5 g/l proved to be appropriate for the cultivation of CHO 2F5 cells on Cytopore I and Cytoline I carriers.

7.3.3 Experiments to increase specific productivity

Temperature shift is described in literature as a method to increase the specific productivity of mammalian cells including CHO cells (61, 62, 63). However the effect is cell line specific and also a reduction in specific productivity following temperature decrease has been reported (61). In the case of CHO 4E10 cells the cultivation temperature was reduced from 37 to 34 °C to increase the specific productivity. The cells showed a different response on Cytoline I and Cytopore I carriers. The specific productivity increased by 50 % from 2.5 to 3.7 $pg*c^{-1}*d^{-1}$ on Cytoline I carriers. In contrast to this on Cytopore I carriers the specific productivity remained unchanged at a level of about 8 $pg*c^{-1}*d^{-1}$ at 37 and 34 °C. CHO 2F5 cells grown on Cytoline I carriers even reduced their specific productivity from 3 to 2 $pg*c^{-1}*d^{-1}$ following temperature reduction from 37 to 33 °C. A further possibility to increase the specific productivity is the addition of sodium butyrate to cell cultures (64, 65, 66). However the substance has also been linked to induction of apoptosis and reduced glycosylation (64, 65). During the cultivation of CHO 2F5 cells on Cytopore I carriers sodium butyrate concentrations of 0.075 to 1 mM were applied. The two lowest concentrations of 0.075 and 0.15 mM did not increase specific productivity but seemed to have a stabilising effect on the parameter. Sodium butyrate concentrations of 0.25 mM and higher increased the specific productivity from 4 to about 5.5 $pg*c^{-1}*d^{-1}$ however at the same time they also caused cell detachment and therefore led to a 20 % reduction in volumetric productivity.

7.3.4 Development of an aeration system for cell cultivation on Cytopore I carriers

Microcarrier based perfusion systems generally operate with very high cell concentrations. During the CHO 2F5 cultivation on Cytopore I a maximum concentration of $1.3*10^7$ cells/ml was achieved. This is in good accordance to literature data where cell concentrations of $1.2*10^7$ to $2*10^7$ cells/ml have been reported for the cultivation of CHO cells on Cytopore I carriers (67, 68). These cell concentrations require high oxygen transfer rates. While in small scale up to 2 liters bubble free membrane aeration has been used successfully (61) for large scale processes only direct sparging is applicable. To increase the k_La value at a given reactor configuration usually the bubble size is reduced to increase the surface area of the gas bubbles. This works well in the FBR as the Cytoline I carriers have a density of 1.34 g/ml and are therefore unaffected by the small bubbles. The situation is however different for Cytopore I carriers that have a significantly lower density of only 1.03 g/ml as they are designed for the use in STRs. Gas bubbles attach to these carriers and transport them to the liquid surface. Additionally small bubbles form a more stable foam layer and therefore effectively remove carriers and the attached cells from the cultivation system. The most efficient available sparger had a pore size of 0.2 µm. Due to the small bubble size it could not be used in Cytopore I cultures. The standard aeration device for batch and fedbatch cultures was a 1 mm tube connector that did not cause foaming however in this case the k_La of the system was rather low and the cells could only be provided with oxygen up to a concentration of about $4*10^6$ to $5*10^6$ cells/ml. After experiments with numerous different spargers aeration of Cytopore I cultures was done using a ring sparger with fourteen 1 mm holes. This device provided enough oxygen to support $1.3*10^7$ cells/ml although it was necessary to use high gas flow rates of up to 2 vvh. To further increase the cell concentration in Cytopore I cultures the development of more efficient aeration systems will be necessary also scale up will be more viable if lower gas flow rates can be used.

7.3.5 Comparison of the carrier cultivation systems

All four tested CHO cell lines had a higher specific productivity on Cytopore I carriers than on Cytoline I carriers. The specific productivity on Cytopore I was about equal to suspension culture in the case of CHO HB617 it was even 3 fold increased. The growth rate during the exponential phase was similar on both carriers as well as in suspension culture. Cytopore I carriers can be used in stirred tanks which means that existing vessels can be adapted to a perfusion process. The retention of Cytopore carriers can be done using gravity settlers which is a robust and scaleable method. One issue that still needs optimisation is the aeration of Cytopore based carrier cultures where foaming needs to be avoided at the same time the oxygen transfer rate needs to be increased to reduce the necesarry gas flow rates.

The Cytoline I based cultivation in a fluidised bed reactor is technically the more mature process as all problems concerning carrier retention and oxygenation have been solved. However cell cultivation on Cytoline I carriers seems to be more delicate than on Cytopore I carriers. In general the specific productivity on Cytoline I carriers was lower than on Cytopore carriers. Moreover for two of the four tested cell lines (CHO 2G12 and CHO 2F5) a dramatic decline of the specific productivity was observed during the initial phase of the cultivation. This effect is cell line specific as the two other cell lines (CHO HB617 and CHO 4E10) showed a constant specific produc-

tivity over 25 to 30 days at comparable cell concentration which makes oxygen limitation an unlikely reason for the decline in specific productivity. Also nutrient limitation concerning glucose and amino acids can be excluded. There are hints that the reduced specific productivity might be linked to high perfusion rates as the parameter increased when the perfusion rate was reduced during CHO 2F5 cultivation. In general medium optimisation is an important issue in perfusion processes as it allows to increase the product concentration due to reduced flow through rates (46).

7.4 Use of LDH measurement for viability determination

To assess cell viability during the cultivation on microcarriers LDH was tested as indicative parameter for membrane disintegration. The LDH concentration in culture supernatant samples frozen at –80 °C was stable. After 300 days of storage the enzymatic activity was still 92 % of day 1. Cell viability determined by trypan blue exclusion and the amount of released LDH showed a correlation coefficient of 0.99 when both parameters were analysed during batch cultivation of CHO cells in spinner flasks. The LDH content per cell was determined to be $7.54*10^{-7}$ U/cell for CHO 2F5 cells. Which is significantly lower than the $3.04*10^{-6}$ U/cell found for CHO K1 cells by Haslam et al. (57). However the amount of LDH released per dead cell varies with not only with celltype but also with cultivation conditions. The data for Vero cells range between $5*10^{-7}$ U/cell (57) and $2*10^{-6}$ U/cell (56).

When CHO cells were cultivated on Cytoline I carriers the analysis of the LDH concentration gave inconclusive results. During the growth phase the LDH concentration increased in parallel with the cell number indicating a constant specific LDH release of the cells and therefore also constant viability. A decrease in specific productivity which might be a hint for reduced viability during the growth phase coincided with a 75% increase in specific LDH release. However during the stationary phase the specific productivity decreased dramatically from 3 to less than 0.5 pg*c-1*d-1 and the specific LDH release declined almost in parallel to this development thus the concentration of the enzyme did not indicate a reduced viability.

The LDH concentration was also tested during the cultivation of CHO 4E10 and CHO 2F5 cells on Cytopore prototypes with different charge concentration. For CHO 4E10 cells it was evident that the carrier prototype which supported the highest cell count (0.48 meq/g) also led to the highest cell viability. With increasing charge concentration the maximum cell counts on the carrier prototypes declined and the specific LDH concentration increased. For CHO 2F5 cells no clear correlation between cell count and viability was found. The charge concentration for optimal cell growth did not coincide with the one for optimum viability. As shown in Figure 127 a charge concentration of 1.37 meq/g caused about 10 % viability increase compared to 0.76 and 1.8 meq/g. The cell viabilities were followed during cultivation time. While on day 4 the charge optimum was already visible the viability difference between the individual experiments was still small amounting to about 3 %. However with increasing cultivation time the viability distribution on the different Cytopore prototypes remained the same but the difference increased to about 10 %. This development seems plausible. Thus in the Cytopore experiments that were conducted as semicontinuous perfusion the LDH concentration was useful as indicative parameter for cell viability.

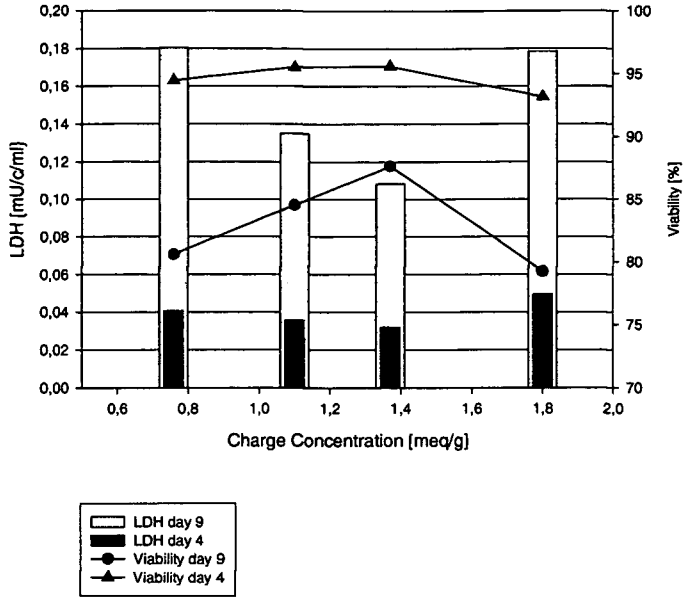


Figure 127: LDH concentration and viability of CHO 2F5 cells on Cytopore prototypes

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