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Cornelia Kasper · Verena Charwat
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Cell Culture Technology

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Cornelia Kasper
Verena Charwat
Antonina Lavrentieva
Editors

Cell Culture Technology

Editors
Cornelia Kasper
Department of Biotechnology
University of Natural Resources & Life Sciences
Vienna, Austria

Verena Charwat
Department of Biotechnology
University of Natural Resources & Life Sciences
Vienna, Austria

Antonina Lavrentieva
Institute for Technical Chemistry
Gottfried Wilhelm Leibniz Universität
Hannover, Germany

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Preface

Cell culture technology has rapidly evolved over the past half century and thus cannot be "limited" any more to the classical fermentation and animal production cell line culture technologies. Nowadays, methods comprise also of modern 3D techniques for cell expansion and/or differentiation. The approaches for tissue engineering and cell-based therapies have woken the demand of cultivation strategies beyond standard cell culture methods. These include the development and establishment of suitable protocols for cultivating cells – especially primary cells – under physiological conditions.

This textbook is structured into 10 different chapters: brief history of the developments in mammalian cell culture technology until today including stem cell cultivation; introduction to relevant equipment and consumable materials as well as standard techniques used in cell culture labs; outline on requirements, developments, and current status in media design; summary of tumor cell line and tissue cultivation; introduction of 3D culture techniques for tissue engineering and cell-based testing systems; overview on relevant biomaterials and their characteristics; demonstration of mechanisms and effects involved in cell-surface interaction; definition and discussion on physiological conditions with focus on oxygen in cell culture procedures; overview on different co-culture systems; and an introduction to suitable strategies for automation of cell culture procedures.

This textbook is a unique collection of chapters relevant for modern cell culture technology, providing an excellent overview of the "essentials" and the current paradigms, as well as insights into relevant methods underlying modern cell culture technology. The chapters are based on lectures and teaching material of the authoring teams, thus making the textbook excellently suitable for teaching and to support bachelor, master, and doctoral theses.

Notwithstanding, if the reader already has experience in mammalian cell culture, or he or she is a novice to this field, this book will be a valuable reading, which will deepen knowledge and expand the understanding how modern cell culture works.

We thank all authors of this book who in addition to their daily chores and academic work helped us with their brilliant and outstanding contributions.

Antonina Lavrentieva
Hannover, Germany

Cornelia Kasper
Vienna, Austria
June 2018

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Contributors

Muhammad Ahmad
Guy Hilton Research Centre
Institute of Science and
Technology in Medicine
Keele University, Stoke on Trent, UK
moh1977729@gmail.com

Mohammed Al Zubaidi
Guy Hilton Research Centre, Institute of
Science and Technology in Medicine
Keele University
Stoke on Trent, UK
mohmsc82@gmail.com

Rakad Al-Jumaily
Guy Hilton Research Centre, Institute of
Science and Technology in Medicine
Keele University
Stoke on Trent, UK
r.m.al-jumaily@keele.ac.uk

Samira Azarin
Department of Chemical Engineering
and Materials Science
University of Minnesota
Minneapolis, MN, USA
azarin@umn.edu

Aldo R. Boccaccini
Institute of Biomaterials, Department of
Materials Science and Engineering
University of Erlangen-Nuremberg
Erlangen, Germany
aldo.boccaccini@ww.uni-erlangen.de

Verena Charwat
Department of Biotechnology, University of
Natural Resources & Life Sciences
Vienna, Austria
verena.charwat@boku.ac.at

Andreas Clementi
Department of Biotechnology, University of
Natural Resources and Life Science
Vienna, Austria
andreas.clementi@boku.ac.at

Tina Dale
Guy Hilton Research Centre, Institute of Science
and Technology in Medicine, Keele University
Stoke on Trent, UK
t.p.dale@keele.ac.uk

Rainer Detsch
Institute of Biomaterials, Department of
Materials Science and Engineering
University of Erlangen-Nuremberg
Erlangen, Germany
rainer.detsch@fau.de

Dominik Egger
Department of Biotechnology, University of
Natural Resources and Life Science
Vienna, Austria
dominik.egger@boku.ac.at

Nicholas R. Forsyth
Guy Hilton Research Centre, Institute of Science
and Technology in Medicine, Keele University
Stoke on Trent, UK
n.r.forsyth@keele.ac.uk

Jan Hansmann
Department Tissue Engineering and
Regenerative Medicine (TERM)
University Hospital Wuerzburg
Wuerzburg, Germany
jasmin.hum@fau.de

Ralf Hass
Biochemistry and Tumor Biology Lab,
Department of Obstetrics and Gynecology
Hannover Medical School
Hannover, Germany
Hass.ralf@mh-hannover.de

Reinhard Henschler
Blood Transfusion Services SRC
Zürich/Chur, Switzerland
reinhard.henschler@blutspende-gr.ch

Wei-Shou Hu
Department of Chemical Engineering and Materials Science, University of Minnesota
Minneapolis, MN, USA
wshu@umn.edu

Jasmin Hum
Institute of Biomaterials, Department of Materials Science and Engineering
University of Erlangen-Nuremberg
Erlangen, Germany
jasmin.hum@fau.de

Roland Jacobs
Department of Clinical Immunology and Rheumatology, Hannover Medical School
Hannover, Germany
Jacobs.Roland@mh-hannover.de

Cornelia Kasper
Department of Biotechnology, University of Natural Resources and Life Science
Vienna, Austria
cornelia.kasper@boku.ac.at

F. Kurtis Kasper
Department of Orthodontics, The University of Texas Health Science Center at Houston
School of Dentistry
Houston, TX, USA
Fred.K.Kasper@uth.tmc.edu

Antonina Lavrentieva
Institute of Technical Chemistry
Leibniz University Hannover
Hannover, Germany
Lavrentieva@iftc.uni-hannover.de

Megan Livingston
Department of Orthodontics, The University of Texas Health Science Center at Houston,
School of Dentistry
Houston, TX, USA
Megan.Livingston@uth.tmc.edu

Katharina Meditz
Division of Biological Research
Medical University of Graz
Graz, Austria
katharina.meditz@medunigraz.at

Marwan Merkhan
Guy Hilton Research Centre, Institute of Science and Technology in Medicine, Keele University
Stoke on Trent, UK
merkhanmarwan@yahoo.com

Sofie O'Brien
Department of Chemical Engineering and Materials Science, University of Minnesota
Minneapolis, MN, USA
bluvs002@umn.edu

Yonsil Park
Department of Chemical Engineering and Materials Science, University of Minnesota
Minneapolis, MN, USA
yonsil.park@gmail.com

Beate Rinner
Division of Biological Research
Medical University of Graz
Graz, Austria

Division of Biomedical Research, Core Facility Alternative Biomodels and Preclinical Imaging
Graz, Austria
beate.rinner@medunigraz.at

Judith A. Roether
Institute of Biomaterials, Department of Materials Science and Engineering
University of Erlangen-Nuremberg
Erlangen, Germany
judith.roether@fau.de

Ivo Schwedhelm
Department Tissue Engineering and Regenerative Medicine (TERM)
University Hospital Wuerzburg
Wuerzburg, Germany
ivo.schwedhelm@uni-wuerzburg.de

Rachel Steeg
Guy Hilton Research Centre, Institute of Science and Technology in Medicine, Keele University
Stoke on Trent, UK
rainer.detsch@fau.de

Julia Will
Institute of Biomaterials, Department of Materials Science and Engineering
University of Erlangen-Nuremberg
Erlangen, Germany
julia.will@fau.de

Abbreviations

AFM	Atomic force microscopy
AIA	Activation-induced apoptosis
ALS	Amyotrophic lateral sclerosis
APCs	Antigen-presenting cells
Asn	Asparagine
ATMPs	Advanced therapy medicinal products
ATP	Adenosine triphosphate
BAL	Bioartificial liver
BG	Bioactive glasses
BHK	Baby hamster kidney
BMP	Bone morphogenetic protein
BrdU	Bromodeoxyuridine
CAD	Computer-aided design
CAR-T cells	Chimeric antigen receptor T cells
CD	Cluster of differentiation
CFSE	Carboxyfluorescein succinimidyl ester
CO_2	Carbon dioxide
CoC	Ceramic-on-ceramic
CFU	Colony forming units
CHO	Chinese hamster vary
µCT/nCT	Micro-/nano-computer tomography
DAPI	4′,6-Diamidino-2-phenylindole
DHFR	Dihydrofolatreduktase
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
rDNA	Recombinant DNA
DO	Dissolved oxygen
DPN	Diphosphopyridine nucleotide
EBV	Epstein-Barr virus
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EL	Extra-luminal
ELISA	Enzyme-linked immunosorbent assay
EMEM	Eagle's Minimum Essential Medium
EPI	Epiblast
EPO	Erythropoietin
ESCs	Embryonic stem cells
ETO	Ethylene oxide
FACS	Fluorescence-activated cell sorting
FAD	Flavin adenine dinucleotide
FBS	Fetal bovine serum
FDA	Food and Drug Administration
FEM	Finite element method
FGF	Fibroblast growth factor
FLIM	Fluorescence lifetime imaging microscopy
FMD	Foot-and-mouth disease
FRET	Förster resonance energy transfer
G6PD	Glucose-6-phosphate dehydrogenase
G-CSF	Granulocyte colony-stimulating factor
GCP	Good clinical practice
GFP	Green fluorescent protein
GMP	Good manufacturing practice
GOI	Gene of interest
GVHD	Graft-versus-host disease
HA	Hydroxyapatite
HBSS	Hank's buffered salt solution
HEPES	4-(2-Hydroxyethyl)-1-pipera-zineethanesulfonic acid
HFB	Hollow fiber bioreactors
HPV	Human papilloma virus
HPL	Human platelet lysate
HSCs	Hematopoietic stem cells
ICM	Inner cell mass
IL	Intraluminal
iPSCs	Induced pluripotent stem cells
IMDM	Iscove's Modified Dulbecco's Medium
ITS	Insulin-transferrin-sodium selenite
LC-MS	Liquid chromatography–mass spectrometry
LCL	Lymphoblastoid cell lines

LCST — Lower critical solution temperature
LDH — Lactate dehydrogenase

Mab — Monoclonal antibody
MACS — Magnetic bead-associated cell sorting
MEF — Mouse embryonic fibroblasts
MHC — Major histocompatibility complex
MLC — Mixed lymphocyte culture
MLR — Mixed lymphocyte reaction
MoM — Metal-on-metal
MRI — Magnetic resonance imaging
MSCs — Mesenchymal stem cells, mesenchymal stromal cells
MTT — 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

NK — Natural killer
NSCs — Neural stem cells

O_2 — Oxygen
OCTGT — Office of Cellular, Tissue, and Gene Therapies

PBMC — Peripheral blood mononuclear cell
PBS — Phosphate-buffered saline
PE — Polyethylene
PGE2 — Prostaglandin E2
PGM — Phosphoglucomutase
PID controller — Proportional-integral-derivative controller
PrE — Primitive endoderm
PI — Propidium iodide
PCR — Polymerase chain reaction
PS — Polystyrene
PSCs — Pluripotent stem cells
tPA — Tissue plasminogen activator
PMMA — Poly(methyl methacrylate)
PVC — Polyvinyl chloride

RNA — Ribonucleic acid
RPMI — Roswell Park Memorial Institute

SCID — Severe combined immunodeficient
SDS — Sodium dodecyl sulfate
SEM — Scanning electron microscopy
Ser — Serine
scFv — Single-chain variable fragment
SCF — Stem cell factor
STR — Short tandem repeat

TAA — Tumor-associated antigen
TCP — Tricalcium phosphate
TE — Tissue engineering
hTERT — Human telomerase reverse transcriptase
TGF — Transforming growth factor
TME — Tumor microenvironment
TPN — Triphosphopyridine nucleotide
TPO — Thrombopoietin

UV — Ultraviolet

VEGF — Vascular endothelial growth factor

WST — Water soluble tetrazolium
7AAD — 7-Aminoactinomycin
2D — Two-dimensional
3D — Three-dimensional

Cell Culture Bioprocess Technology: Biologics and Beyond

Sofie O'Brien, Yonsil Park, Samira Azarin, and Wei-Shou Hu

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What You Will Learn in This Chapter

Scientists have been growing vertebrate cells in culture for over a century. Initially, this cell culture involved placing tissues isolated from animals in a nutrient solution for observation. Eventually, some cells grew out from the tissue explant and began to form cell layers. Over the years, scientists developed a better understanding of the nutritional requirements of cells, established methods to isolate them from tissues and to propagate them, and acquired the ability to use them as tools for conducting research to develop new knowledge on cells and organisms. Ultimately, we learned ways to use them to produce vaccines and medicines.

Cells isolated from human tissues or differentiated from human stem cells are poised to treat organ dysfunctions, repair tissues, and fight cancers. In modern science, various stem cells and differentiated cells, like nerve cells, T cells, and natural killer cells, are isolated, expanded—even genetically modified—and used to treat diseases in patients. Cells are not only major tools of scientific inquiries, but also mediators of medical technology. Animal cells are now used to produce over US $100 bn of therapeutic proteins and viral vaccines.

In this chapter, you will learn how cell culture progressed from a scientist's tool to a major technological medium for manufacturing. You will also learn how a new generation of cell-based therapy is emerging to potentially transform medical technology.

1.1 Cell Culture: A Tool for Science

1.1.1 The Making of Cells as a Scientific Tool

Like any other science, at its beginning cell culture was almost like an art; the skill of practicing it relied much on scientific intuition, experience, inquisitiveness, and perseverance. The cultivation of animal cells originated as tissue culture around the turn of the last century, when an explant of tissue was submerged in the tissue fluid of another animal. As some cells began to grow out of the tissue clump onto the culture surface, one could observe cell behavior for a few days. Subsequently, some cells were isolated and continued to divide for a small number of generations. These primary cells did not grow in culture for long [24]. By the 1930s, scientists were isolating cells of different differentiation lineages, like nerve fibers, fibroblasts, etc. ◘ Figure 1.1 depicts a timeline of the discovery of important cell lines and technologies.

The short duration that primary cells isolated from tissue were maintainable in the lab limited their use in research. By dissecting the cells after they had grown out of a tissue explant on a dish, some cells could be transferred to a new glass surface to grow further. Using this technique, continuous cell lines, among the first of them the mouse L cell [17], were maintained in culture.

Maintaining these early cell lines required continuous passaging. At that time, cells were grown in a balanced salt solution supplemented with serum, ascitic fluid, and chicken embryo extract [63]. To supply the cell culture medium, animal tissue fluid had to be isolated regularly since no microfiltration membrane for medium sterilization was yet available. Imagine the difficulty of maintaining those cultured cells contamination-free! Then came the use of trypsin to dissociate cells from the surface, and trypsin thus replaced dissection as the means of passaging cells, relieving scientists from many lab routines in maintaining cells. The first human cell line, HeLa, was derived from human cervical cancer but still had to be continuously propagated in culture in order to be maintained in the lab [19, 63].

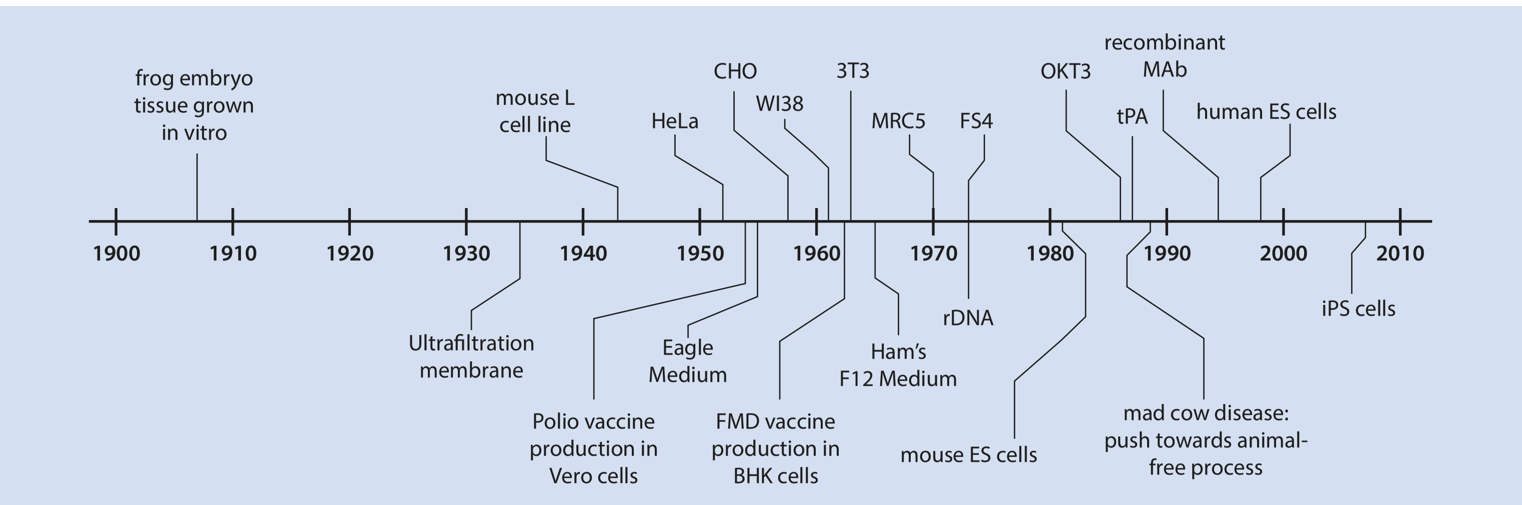


Fig. 1.1 Timeline of events important to the history of cell culture. Abbreviations: *CHO* Chinese hamster ovary, *FMD* foot-and-mouth disease, *BHK* baby hamster kidney, *rDNA* recombinant DNA, *ES cells* embryonic stem cells, *tPA* tissue plasminogen activator, *Mab* monoclonal antibody, *iPS cells* induced pluripotent stem cells

With the ability to passage and expand the cell population, scientists grew interested in studying the cell's nutritional requirements. However, since the beginning of cell culture, autoclaving has been used for sterilizing any liquid, supplies, and equipment. Besides salt solutions, the other components, including tissue fluid nutrient mixtures, could not be easily sterilized because they were heat labile. The commercialization of nitrocellulose membranes for ultrafiltration allowed complex nutrient mixtures to be sterilized by membrane filtration and contributed to the advances in cell research. The development of a chemical nutrient medium consisting of glucose, amino acids, vitamins, and balanced salts in 1955 reduced the burden of isolating tissue fluids [16]. Instead of containing >50% serum or tissue fluids, cell culture medium became >80% basal chemical medium. Another important facilitator of cell culture was the development of cryopreservation. The discovery that animal sperm could be preserved at −79 °C using glycerol as the cryoprotectant [71] led to its use in preserving cultured cells. This alleviated the need for continuous passaging of cells in culture, allowing more researchers to employ cells as a research tool.

1.1.2 Normal Cells, Senescence, and Differentiation

The first established cell lines, e.g., mouse L and HeLa, were derived from cancerous tissues. Efforts to derive cell lines from normal tissues had lagged because those cells underwent senescence. For example, fibroblasts could grow out of chicken embryos and proliferate in culture but quickly lost growth potential after a number of passages. By continuously passaging fibroblasts isolated from a mouse embryo, an apparently normal cell line, 3T3, was derived [80]. 3T3 cells are phenotypically normal and exhibit adhesion dependence and contact inhibition. They do not undergo senescence, but their karyotype is aneuploid, not diploid. Many cell lines from various animal tissues were subsequently isolated, including baby hamster kidney (BHK) [39], Vero [90], and Chinese hamster ovary (CHO) [57], from Syrian hamster, green monkey kidney, and Chinese hamster, respectively. However, unlike 3T3 cells, those cells are not phenotypically normal, and many do not exhibit contact inhibition, nor are they diploidic.

Around the same time, a phenotypically normal human fibroblastic cell strain, WI38, was isolated [26]. These cells were diploid, exhibited contact inhibition, and were anchorage-dependent. But they were not a continuous cell line like 3T3. They underwent senescence after ~40 subculturings. This observation led to what is known as the Hayflick phenomenon, in which normal diploid cells undergo only a limited number of cell divisions before they cease to divide. An exception to the Hayflick limit would not be seen until the isolation of stem cells. Subsequent isolation of primary cell lines MRC-5 [28] and FS4 [85] led to their use for viral vaccine and interferon production, respectively. MRC-5 cells are still used in the manufacturing of many viral vaccines today.

1.1.3 Differentiated Cell Lines

Following HeLa cells, more human cells were isolated from normal or cancerous differentiated tissues. Some cell lines still carried the phenotypic characteristics of the tissue that they were derived from. HepG2 cells derived from hepatocellular carcinoma express many liver markers and secrete albumin [1]. Jurkat cells, a human T lymphocyte cell line isolated

from leukemia, express specific markers of T cells such as CD3, CD4, and CD45 [64]. PC12, isolated from a pheochromocytoma of the rat adrenal medulla, can be induced to form dendrites in culture [21]. These and other differentiated cell lines became valuable tools for biomedical research.

Some cell lines can undergo differentiation in vitro when they are exposed to appropriate signals. For example, a subclone of 3T3 cells, designated 3T3-L1, can be induced to differentiate into adipocytes [20]. These cell lines heralded the idea of employing differentiated cells for in vitro testing and eventually for therapy.

1.1.4 Generating Continuous Cell Lines by Genetic Manipulation

Cell lines were also generated by genetic modification to give them immortality. The HEK293 cell line was derived from human embryonic kidney cells by introducing the adenovirus type 5E1 DNA [62]. The simian virus 40 large T antigen (SV40 Tag) and human papillomavirus 16 (HPV16) E6/E7 were used to derive many different cell types such as duct epithelial cells, hepatocytes, and epidermoid carcinoma cells [33, 58]. Human telomerase reverse transcriptase (hTERT) was overexpressed to derive cell lines by countering the loss of telomerase. These cells retained a stable genotype and critical phenotypic markers, more closely mimicking the physiology of cells in vivo [38, 43, 75].

Immortalized cells enriched the repertoire of cell lines for biomedical research. Some are used in biomanufacturing. HEK293 cells are used for the commercial production of protein C and are also used in the production of adeno-associated virus for gene therapy [12]. They are also the favorite cell line for transient gene expression in the biotech industry for obtaining an investigation quantity of proteins. However, in terms of differentiated functions, the cell lines, whether they are immortalized or derived from tissues, are less biologically relevant because many of the key characteristics of the original tissue are not retained. For example, key cytochrome P450 enzymes are expressed only at very low levels in HepG2 and other hepatoma cell lines, even though they secrete albumin and other liver proteins [61]. In the subsequent era of tissue engineering and regenerative medicine, the cells of choice became stem cells or other primary cells as will be discussed later in this chapter.

1.1.5 Industrial Cell Culture

1.1.5.1 Cell Culture Processes in Classical Industrial Biotechnology

From its beginning, cell culture research was purposeful, with possible applications in mind. Initial cell isolation was partly for observing tumors and malignancy. Soon after their isolation, HeLa cells were used to study the replication of polio virus [63]. Some viral vaccines had been traditionally grown in animals or in chicken embryos within the egg. By the 1940s, foot-and-mouth disease (FMD) virus was produced using primary calf kidney cells [76]. So were many other viral vaccines being produced in primary cells derived from animal tissues, including the polio vaccines in the 1950s [4]. Once scientists started to grow continuous cell lines and serially passage them in culture, vaccines also began to be produced in roller bottles using continuous cell lines, including BHK cells for FMD [49] and Vero cells for polio [34].

In addition to virus production, many differentiated cells are capable of producing functional biologicals. We thus also saw various molecules with beneficial biological functions produced by cultured human cells in the 1970s, such as interferon produced by FS4 cells [85]. However, soon the use of human cells for the production of cytokines was replaced by recombinant DNA (rDNA)-based methods.

1.1.6 Animal Cell Culture in the rDNA Era

The invention of recombinant DNA technology [13] changed the landscape of cell culture technology. Several molecules that were previously isolated from tissues, such as insulin and human growth hormone, were soon produced in *Escherichia coli*. However, many proteins of therapeutic value required posttranslational modifications, such as glycosylation and multiple disulfide-bond formation, that are beyond the natural capabilities of *E. coli* and other microorganisms. These proteins must be produced using cells isolated from mammals.

The early products of recombinant therapeutic proteins, including tissue plasminogen activator (tPA) (FDA approval 1987), erythropoietin (EPO) (1989), and Factor VIII (1992), were native proteins. tPA helps dissolve blood clots in stroke patients, EPO stimulates red blood cell generation, and Factor VIII is used to treat hemophiliac patients. Owing to hybridoma technology [35], starting a decade later and continuing to this day, antibodies became the prevailing class of therapeutic agent. The hybridoma cell was the cell product of fusion between a producing cell of a specific antibody that could hardly proliferate in culture and a myeloma cell. The fused hybridoma cell acquired the specific antibody-producing capability of a B cell and the unlimited proliferation potential of a myeloma. Once a hybridoma cell line producing antibody against a specific antigen was generated, the antibody gene could be cloned to generate a producing cell line using a preferred host cell.

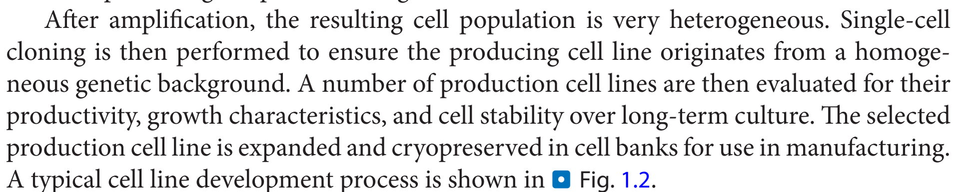
The emergence of antibodies as medicine changed the drug discovery process from the traditional screening-based approach to a design-based process. The discovery of classical bioactive compounds relied on screening a large collection of microorganisms to detect the target biological or binding activity. With recombinant antibody technology, once a target is identified, an antibody can be obtained quickly by immunizing an animal with the antigen. The capability of the antibody to bind to the target molecule is never in doubt. Thus, the success rate in developing antibody products is much higher than the screening of traditional natural products produced by microorganisms. Such design-based approaches to treating diseases fueled the expansion of protein therapeutics in human health care.

1.2 Cell Culture Bioprocess Technology

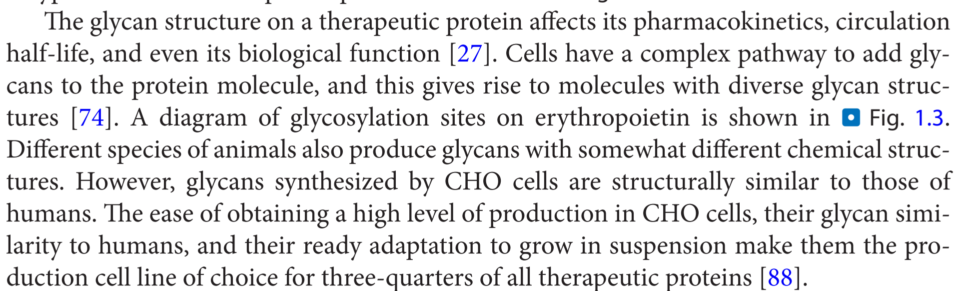
1.2.1 Construction of the Production Cell Line

Only a small number of host cell lines are used for therapeutic protein production: mouse myeloma (SP2/0, NS0), BHK cells, HEK293 cells, and CHO cells. Among these cell lines, CHO cells have become the predominant host cell for producing protein therapeutics over the years. Auxotrophic mutants, or mutants with additional nutrient requirements, cannot

be easily isolated from mammalian cells, as they are diploid and a mutation in one allele is often masked by the other allele. However, for reasons not entirely clear at the time, CHO cells were prone to generate various mutants. They thus became one of the favorite cell lines for cytogenetic research. It was found that a segment of mammalian genome that contains the DHFR gene could be amplified by hundreds of copies upon exposing CHO cells to a very high concentration of a metabolic inhibitor of DHFR [52]. This research was then applied further in a CHO mutant that was partially defective in DHFR, where, upon the introduction of a plasmid vector containing the DHFR gene and its integration into the genome, DHFR could be amplified to hundreds of copies after exposure to a high level of the DHFR inhibitor methotrexate [31]. With a gene of interest (GOI) adjacent to DHFR in the plasmid, the product gene can get amplified to tens or even hundreds of copies, and cell lines producing the product at high levels can be obtained.

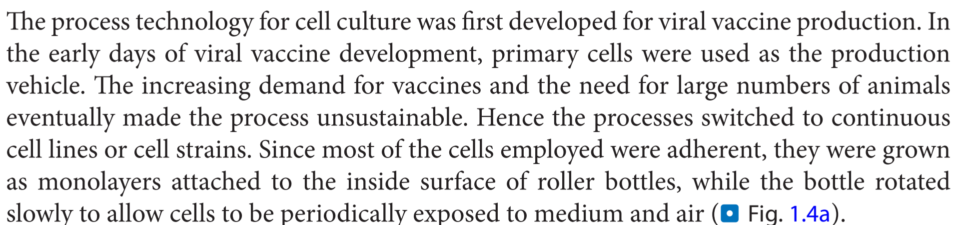


After amplification, the resulting cell population is very heterogeneous. Single-cell cloning is then performed to ensure the producing cell line originates from a homogeneous genetic background. A number of production cell lines are then evaluated for their productivity, growth characteristics, and cell stability over long-term culture. The selected production cell line is expanded and cryopreserved in cell banks for use in manufacturing. A typical cell line development process is shown in ◘ Fig. 1.2.



The glycan structure on a therapeutic protein affects its pharmacokinetics, circulation half-life, and even its biological function [27]. Cells have a complex pathway to add glycans to the protein molecule, and this gives rise to molecules with diverse glycan structures [74]. A diagram of glycosylation sites on erythropoietin is shown in ◘ Fig. 1.3. Different species of animals also produce glycans with somewhat different chemical structures. However, glycans synthesized by CHO cells are structurally similar to those of humans. The ease of obtaining a high level of production in CHO cells, their glycan similarity to humans, and their ready adaptation to grow in suspension make them the production cell line of choice for three-quarters of all therapeutic proteins [88].

1.2.2 Process Engineering of Cell Culture



The process technology for cell culture was first developed for viral vaccine production. In the early days of viral vaccine development, primary cells were used as the production vehicle. The increasing demand for vaccines and the need for large numbers of animals eventually made the process unsustainable. Hence the processes switched to continuous cell lines or cell strains. Since most of the cells employed were adherent, they were grown as monolayers attached to the inside surface of roller bottles, while the bottle rotated slowly to allow cells to be periodically exposed to medium and air (◘ Fig. 1.4a).

To further scale up, some cells were adapted to grow in suspension without surface attachment, such as BHK cells [76]. They could then be cultivated in stirred tank reactors with oxygen being supplied by bubbling air through the medium. This strategy of suspension growth was later adopted for the production of recombinant proteins. However, many primary cells and cell lines, such as Vero monkey kidney cells used in polio virus production, could not be grown in suspension. Scale-up of those processes faced tremendous challenges. The invention of microcarriers allowed cells to attach to the external surface of those small beads and then be suspended in a stirred tank reactor [83]. This innovation facilitated the manufacturing of many human and veterinary vaccines.

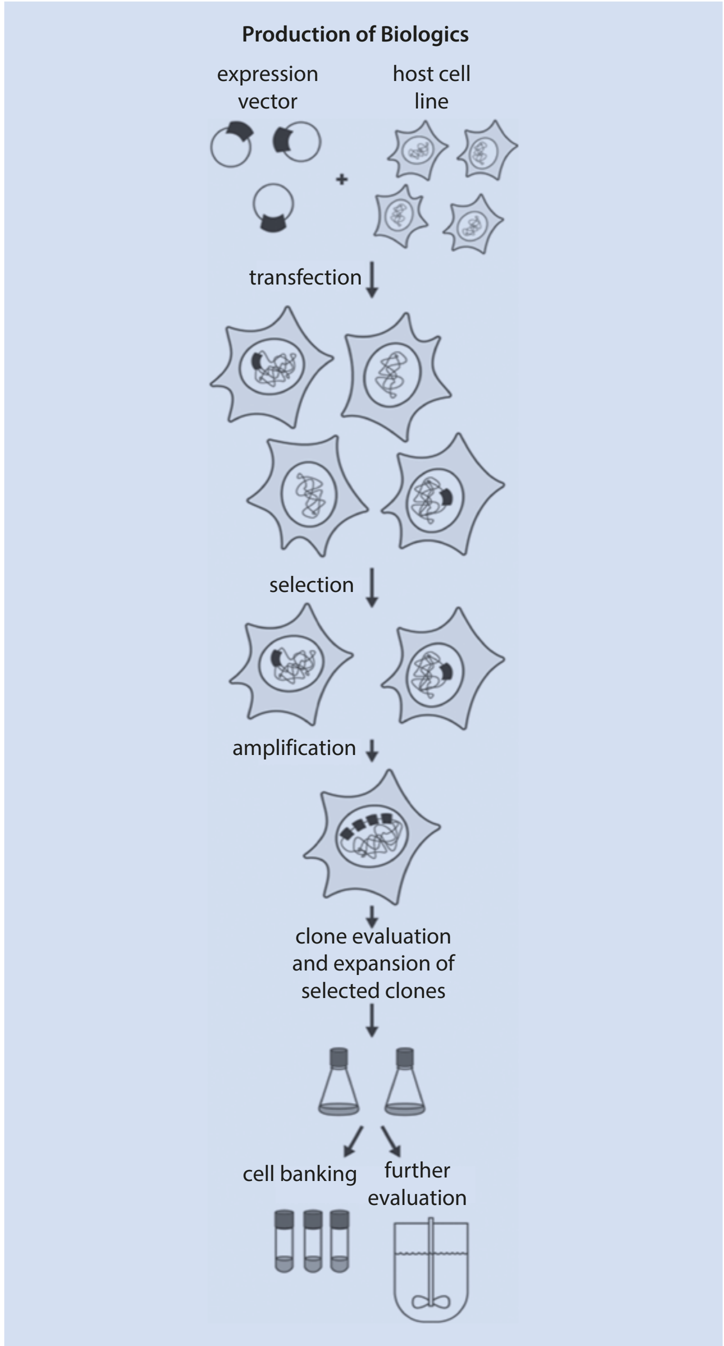


Fig. 1.2 Cell line development for the production of biologics. An expression vector containing a product gene of interest and a selection gene is transfected into a host cell line. A selection pressure is then applied to force integration of the vector into the genome. Increasing levels of the selection agent are used to amplify the vector containing both the selection gene and the gene of interest within the genome to get a high-producing cell line. The cells are then subcloned, cell banks are made, and clones are evaluated for their productivity, stability, and growth characteristics

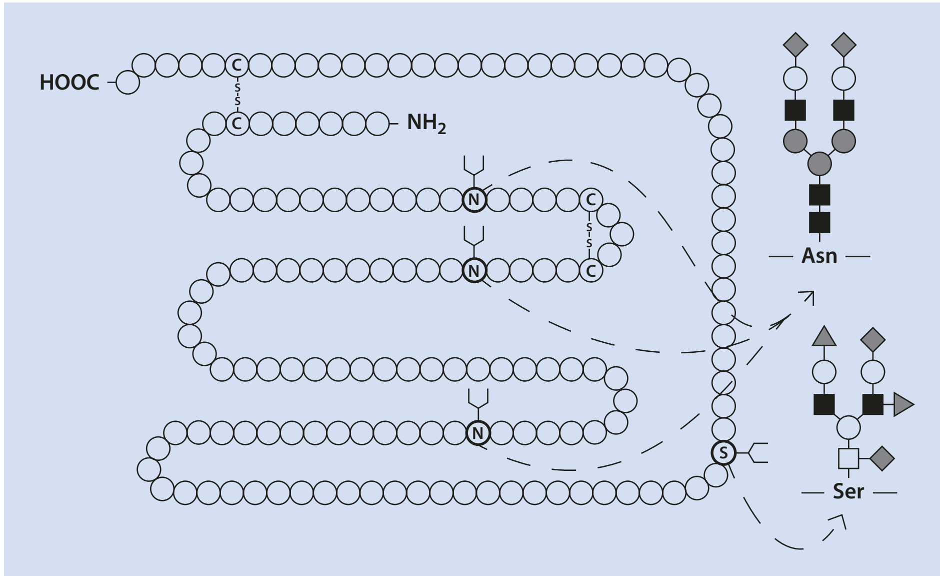


Fig. 1.3 Glycosylation sites on erythropoietin. Erythropoietin has four sites for glycosylation, three N-glycosylation sites on asparagine (Asn), and one O-glycosylation site on serine (Ser). These sugar glycans can influence many properties of the resulting therapeutic molecule, such as its activity, half-life, and efficacy

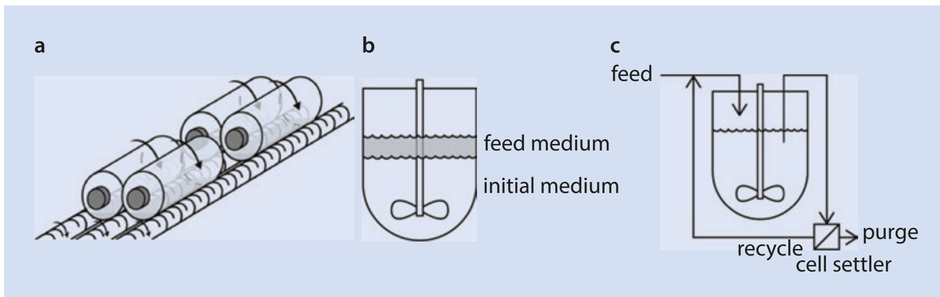


Fig. 1.4 Reactors for cell culture. **a** A roller bottle setup. Cells are grown on the roller bottle surface, and the bottles are rotated at ~4 rpm such that the cells maintain periodic contact with the medium. **b** A fed-batch culture. Cells are grown in a bioreactor and feed medium is gradually added over the course of the culture. **c** A continuous/perfusion culture. Cells are grown in a reactor and medium is being fed continuously. A collection stream continuously removes medium containing cells, product, and other medium components. The effluent is then put through a cell settler to separate the cells from the product. The purge is collected for protein purification, while the recycle stream delivers concentrated cells back to the reactor

The dose of vaccines for immunization is very small. The number of doses that can be produced per culture volume is large compared to therapeutic protein products. Bioreactors used in vaccine manufacturing are thus relatively small and operated in batch mode. For protein therapeutics, especially antibody products, the dose is high. The cell and product concentration per reactor volume has to be high to increase the number of doses that

can be produced in each manufacturing run. Because of cells' intolerance to high levels of nutrients and osmotic pressure, the concentration of nutrients in the medium is low; thus the final concentrations of cells and product achievable in a batch operation are also low. Fed-batch operation became commonly practiced for protein production. The culture is operated by starting at ~75% of total culture volume; subsequently, concentrated nutrient feed is gradually added to the full capacity of the reactor to achieve high concentrations of cells and product (Fig. 1.4b).

Some cell culture products, such as the native form of Factor VIII, are unstable in the culture medium. They are produced in continuous cultures with a short holding time. To avoid washing out cells, a cell retention or cell separation device is used to discharge a dilute cell stream and recycle a concentrated cell stream back to the reactor. This operation of a continuous cell culture with cell retention is commonly called perfusion (Fig. 1.4c).

Medium is the heart of cell culture. The drive to better understand the nutritional needs of cultured cells turned to developing serum-free media in the 1970s [7, 22]. In addition to glucose, amino acids, vitamins, nucleoside precursors, lipids, and bulk salts (potassium phosphate, sodium chloride, etc.), cells also require trace elements (e.g., selenium and zinc ions), carrier proteins (e.g., transferrin, albumin), and some growth factors. Despite the progress, animal serum continued to be used in the manufacturing of vaccines and therapeutic proteins until the outbreak of bovine spongiform encephalopathy in the United Kingdom raised grave concerns about using animal serum in biomanufacturing. In modern cell culture processes, compounds derived from animals are not permitted in the medium, so chemically defined media is used in manufacturing. With a chemically defined medium, one aims to enhance the controllability of the process and the product quality.

1.2.3 Cell Culture Biomanufacturing

The volumetric capacity of a cell culture manufacturing plant varies over a very wide range. A vaccine plant is usually small but often produces millions of doses annually. Some antibodies are produced in thousands of kilograms each year. A manufacturing plant for those products has hundreds of tons of volume capacity, and thus the capital investment for its construction is huge. To mitigate the financial risk, many pharmaceutical companies have begun to employ contract manufacturing organizations (CMOs) located in various parts of the world. It is becoming common that a product is manufactured in multiple sites around the globe. To reduce capital investment, some plants are designed to use plastic disposable bioreactors instead of a large stainless steel stirred tank. This is possible because the power input for a cell culture stirred tank reactor is much lower than that in microbial fermentation. However, a plastic bioreactor cannot sustain the high mechanical stress caused by mechanical agitation in scaling up. Thus, the size of a disposable stirred tank reactor is limited. Nevertheless, by using a disposable bioreactor, the cost of plant construction is relatively low and the time for construction is short. Hence emerges a trend of distributed manufacturing, using a number of small facilities based on single-use bioreactors located in different regions to produce the goods. This new way of manufacturing may see its adoption in the manufacturing of cell-based products that we will discuss in the following section.

1.3 Functional Cell Lines

While the biopharmaceutical industry underwent an unprecedented expansion, a fundamental shift was happening in biomedical research. Various growth factors and cytokines became easily attainable through rDNA technology. Parallel to the boom in the biopharmaceutical industry were the advances in analytical technology (PCR, mass spectrometry, microscopy, high-resolution chromatography, etc.). An expansion of the biomedical service industry and its large repertoire of biochemical reagents greatly increased our capabilities for culturing cells. Primary cells that could be isolated from normal human tissues and cultured in vitro extended from fibroblasts and epithelial cells to many other differentiated cell types such as endothelial cells, keratinocytes, melanocytes, mononuclear cells, smooth muscle cells, pre-adipocytes, etc. The primary tissue cells isolated from tissues typically retain many tissue-specific activities. They may be exploited for use to repair or augment tissue functions. However, most differentiated cells have very limited proliferative potential and show phenotypic instability in culture. For many applications the restricted cell source will limit their potential. In contrast, various stem cells offer the possibility of a virtually unlimited cell supply.

1.3.1 Stem Cells

Stem cells are marked by two characteristics: the ability to self-renew and the potential to differentiate into particular cell types in vivo [87]. They are classified by their differentiation potential: totipotent, pluripotent, and multipotent cells. Totipotent cells can become any type of cell including extraembryonic tissues. Cells that can become any type of cell in all three germ layers of ectoderm, mesoderm, and endoderm are pluripotent (i.e., embryonic stem cells, induced pluripotent stem cells) [48]. Multipotent stem cells can differentiate into multiple types of cells in a limited number of cell lineages. For example, hematopoietic stem cells and mesenchymal stem cells are multipotent [87]. These multipotent stem cells are seen in their home tissue in adults. They may be quiescent in their niche and begin to expand and differentiate only when needed. In their in vitro isolation and expansion, the chemical and physical cues in culture make them enter a proliferative state. They tend to have limited proliferative potential, and after some generations they lose their renewal and differentiation capacity.

1.3.1.1 Hematopoietic Stem Cells and Mesenchymal Stem Cells

Bone marrow cell transplantation has been practiced for over three decades, taking advantage of the stem cells or progenitor cells in the bone marrow to repopulate blood cells in the recipient. Hematopoietic stem cells have been isolated from bone marrow, peripheral blood, and umbilical cord blood. They can differentiate into all types of blood cells. There is not a single molecular marker exclusively expressed in HSCs. They are usually identified by the expression or absence of surface markers (Lin-, CD34+, CD38-, CD90+, CD45-, Flt3+, CD7-, CD10-). A combination of growth factors, such as stem cell factor (SCF), thrombopoietin (TPO), and granulocyte colony-stimulating factor (G-CSF) are employed to induce their population expansion in culture.

Mesenchymal stem cells (MSCs) were initially isolated and expanded in culture from adult bone marrow [8]. They were initially known to be adherent fibroblast-like cells

which help to maintain hematopoietic cells in the bone marrow [40]. MSCs are a heterogeneous cell population, mostly consisting of cells with common features in their surface markers including CD90$^+$, CD105$^+$, CD73$^+$, CD14$^-$, CD34$^-$, and CD45$^-$. The classical definition of MSCs are the cells that have moderate self-renewal capacity and are multipotent, with the ability to differentiate mainly into osteogenic, chondrogenic, and adipogenic cells [25].

As MSCs can be found in nearly all tissues, more efforts were expended to identify and localize the cells in situ. It has been proposed that MSCs are pericytes that reside around all blood vessels, and which are in close contact with the basement membrane and surrounding endothelial cells that comprise the microvasculature [9, 70]. Isolated pericytes change into a MSC phenotype depending on the microenvironment or culture conditions. They share several cell surface markers, such as NG2 and CD146, with isolated MSCs [14]. As MSCs also have the capacity to produce a large number of bioactive molecules, their immunomodulatory and trophic properties draw more attention as medicinal signaling cells.

1.3.1.2 Human Embryonic Stem Cells (hESCs)

In early embryo development, after fertilization, cleavage of the zygote forms the morula, and the embryo further develops into the blastocyst composed of the trophectoderm (TE) and inner cell mass (ICM). The TE is the outer layer of the blastocyst and forms extraembryonic ectoderm and placenta in later stages. The ICM gives rise to epiblast (EPI) and primitive endoderm (PrE or hypoblast) layers. The epiblast contributes to all three germ layers: ectoderm, mesoderm, and endoderm. PrE contributes to the extraembryonic endoderm and yolk sac. Stable mouse embryonic stem cells were first derived from ICM in 1981 [18]. Two laboratories derived human embryonic stem cell (hESC) lines in 1998 [65, 79]. hESC lines were derived from culturing gonadal ridges and mesenteries containing primordial germ cells (5–9 weeks postfertilization).

These cell lines had the capability to self-renew in culture and were pluripotent; thus they could differentiate to cell types of all three germ layers. They were karyotypically normal when grown on mouse embryonic fibroblasts (MEF) feeders and formed teratomas when grafted onto severe combined immunodeficient (SCID) mice.

Various approaches have been utilized to optimize the culture conditions of hESCs. hESCs can be maintained in feeder-free environments with the support of extracellular matrices such as Matrigel, fibronectin, and vitronectin [2]. The signaling pathways of BMP, Wnt, and Nodal play key roles in maintaining pluripotency. Cocktails of signaling molecules, including bFGF, activin A, and TGFb1, are used in the feeder-free culture [84].

1.3.1.3 Induced Pluripotent Stem Cells (iPSCs)

hESCs were derived from fertilized human eggs, and thus their use is ethically controversial. The reprograming of adult somatic cells into an undifferentiated embryonic state alleviated the ethical concerns. These cells are called induced pluripotent stem cells (iPSCs) and were derived from both mouse [78] and human [77, 91] adult cells. Upon the introduction of four exogenous genes (OCT4, SOX2, KLF4, and c-MYC (OSKM)), cells proliferate, bypass apoptosis and cell senescence, lose somatic cell characteristics, and undergo a metabolic shift for faster energy generation [78]. The reprogramming is stochastic; only a very small fraction of OSKM-transduced cells acquire fully activated pluripotency [69]. The iPSCs have similar potentials to differentiate to cells of different lineages as ESCs.

The reprogramming of adult somatic cells has been extended to directing one cell type to another lineage [32]. Instead of ascending to the ESC-like state, partially reprogrammed cells were differentiated directly to cells of interest by transducing them with different combinations of gene factors and culturing them in directed differentiation culture medium [93]. This approach has been used to reprogram somatic cells to beta cells, cardiac cells, neurons, hepatocytes, etc. [30, 41, 42, 73, 92].

1.3.1.4 Directed Differentiation of Stem Cells

Pluripotent and multipotent stem cells can be guided by chemical and physical cues to differentiate to a particular lineage. For hematopoietic stem cells (HSCs), SCF and TPO are essential for maintenance and differentiation. In the presence of SCF, TPO, G-CSF, Flt-3 ligand, and interleukin-6 (IL-6), the HSCs isolated from bone marrow can be directed to myeloid lineage cells. Increasing Delta ligand concentration shifts HSCs to lymphoid cells. Using conditions that mimic in vivo embryo development, PSCs were guided to differentiate to the hepatic lineage. A stagewise exposure to growth factors guided them sequentially through endoderm, early, and late hepatic lineage [23, 68, 86]. Similar conditions were used to direct PSCs to the endoderm lineage; then a different set of signaling molecules were used to guide cells to the pancreatic lineage [15]. In addition to endoderm, PSCs have also been differentiated to mesodermal cells. By co-culturing ESCs with OP9 stromal cells and sorting for CD73, mesenchymal precursors were generated and further differentiated into chondrocytes, osteocytes, and myoblasts [6].

Hematopoietic cells can also be generated from PSC-derived mesoderm precursors. Differentiating populations progressed from a pluripotent state to Flk-1$^+$ or PDGFR$^-$ mesoderm to a yolk sac hematopoietic stage (KDR+, CD31+, and CD34+) [51]. IL3, IL6, and BMP4 promoted CD45$^+$ hematopoietic cell differentiation [10]. Co-culture with mouse OP9 stromal cells or the yolk sac endothelial cell line C166 gave rise to CD34$^+$ hematopoietic-like cells [84].

In general, the differentiated cells of different lineages obtained by in vitro differentiation are not the equivalent of their adult counterparts; rather, their differentiation status is closer to that of the fetal state. Our understanding of the mechanisms of embryonic development to a specific tissue or cell type is still limited.

1.4 Emerging Technology Enabling Regenerative Medicine

1.4.1 Tissue Engineering

With the increased capability of isolating and culturing functional differentiated cells, cell culture applications have extended to tissue engineering. The basic idea of tissue engineering is to combine cells, bioactive molecules, and/or synthetic materials as the physical support or scaffold to develop biological substitutes of tissues for repair or even regeneration [36]. Primary chondrocytes were used for the construction of cartilage mimics [44, 60]. Cultured keratinocytes and fibroblastic cells were placed on a synthetic scaffold as a tissue-engineered skin substitute [67]. Primary porcine hepatocytes were cultured in a hollow-fiber bioreactor as an extracorporeal bioartificial liver (BAL) for treating acute liver failure and acute-on-chronic liver failure [45, 53, 54].

A common issue in different applications of tissue engineering or regenerative medicine is the source of functional differentiated cells. For ex vivo *or* extracorporeal applications,

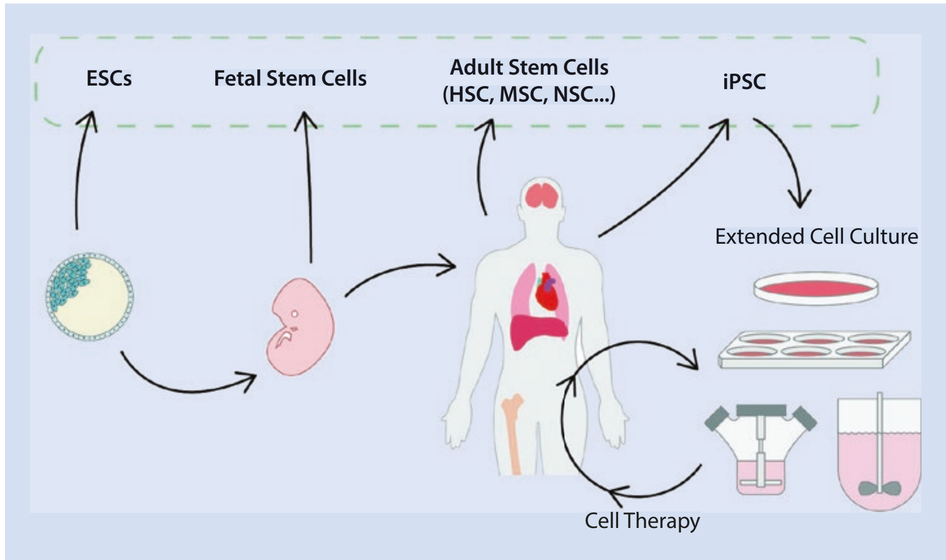


Fig. 1.5 Sources of stem cells. Abbreviations: *ESCs* embryonic stem cells, *HSC* hematopoietic stem cell, *MSC* mesenchymal stem cell, *NSC* neural stem cell, *iPSC* induced pluripotent stem cell

a xenogeneic source may be acceptable. However, for implantation or transplantation applications, a human tissue source must be used and the source will be scarce. Stem cells or cells derived from direct reprogramming can become the cell source for in vivo and in vitro applications as they can be expanded and guided to different cell types. Figure 1.5 shows the various sources of stem cells for research and engineering purposes.

1.4.2 Cell Therapy

Various stem cells including MSCs, PSCs, and NSCs (neural stem cells) have been explored for the treatment of a broad spectrum of disease conditions (for reviews see [47, 59, 81, 82]). PSCs, with their potential as virtually unlimited cell sources, are of particular interest. hESC-derived oligodendrocyte progenitors were explored for spinal cord injury [66]. hESC applications in the eye, pancreas, Parkinson's disease, and heart failure have been investigated in clinical trials [55, 81]. However, because of the potential of teratoma formation from transplanted pluripotent stem cells, by far MSCs still account for the largest number of clinical trials, including acute myocardial infarct, graft-versus-host disease (GVHD), and osteoarthritis (▶ www.clinicaltrials.gov). The clinical potential of MSCs extends beyond their multipotency to immunomodulatory and trophic activities. In those types of treatments, the transplanted cells are present only transiently in the host for a few days, but they induce immunomodulation through suppression of activated T cell proliferation, increase regulatory T (Treg) cells, and influence dendritic cell maturation.

Another type of adult stem cells, neural stem cells (NSCs), are used in a number of clinical trial applications. Fetal neural stem progenitors are being explored for amyotrophic lateral sclerosis (ALS) and stroke. Neural stem cells are being studied for treating spinal cord injury and dry macular degeneration [81].

Gene therapy using autologous cells for treating congenic diseases has been explored for many years. The genetically defective autologous cells are isolated, genetically manipulated to "correct" the defect, and then transplanted to the patient. Gene editing was done to treat genetic hematological disease such as sickle cell disease and thalassemia [5]. Other cells were cultured ex vivo and genetically modified to express cytokines, Fc receptors, and chimeric tumor antigen receptors for cancer immune therapy [11]. T cells were engineered to express a chimeric T cell receptor that has a single-chain variable fragment (scFv) with a high affinity for tumor-associated antigen (TAA), allowing these chimeric antigen receptor T (CAR-T) cells to recognize and kill tumor cells [37].

The success of cell therapy faces several common challenges: cell isolation and/or cell expansion to acquire a large number of cells, engraftment of cells at the target site, and conciliation between autologous (from patient) and allogeneic (from external donor) cell sources.

1.5 Engineering Challenges/NextGen Biomanufacturing

1.5.1 Process Technology for Protein and Viral Biologics

Cell culture processes for the production of biologics are now a robust manufacturing technology. However, they are also facing new challenges to meet societal demands. The protein medicines that used to be privileged drugs for the Western world must be made available to treat patients in less developed countries. The high price of those medicines needs to be reduced to make them available to those in need who currently cannot afford them. In the manufacturing of vaccines, the lag time in ramping up manufacturing capacity and the resulting delay in vaccine distribution to the affected regions in times of pandemics is a grave concern. Although these issues are very much affected by social, economical, and political conditions, the solution will require engineering innovations that enhance productivity, boost the controllability of product quality, and reduce the cost of goods. With the advances in genome engineering, we will likely see fundamental changes in production cell line construction. The tradition of empiricism will give way to systems design-based approaches. The engineering of the product protein will not only enhance its binding properties to the target but also optimize its pharmacokinetic characteristics, maximize its stability, and improve its downstream recovery efficiency. We will engineer the host cell to control its metabolism, which will empower us to modulate the glycosylation pattern of the product.

1.5.2 Emerging Cell Therapeutics

In the past few decades, cell culture evolved from scientific and laboratory exploration to process technology and then to the mature manufacturing enterprise it is today. A challenge in the next decade will be a similar transformation for cell-based therapeutics, from a clinical or laboratory practice to manufacturing technology. In the process of transformation, one aims to make the process robust, the product quality consistent, and the cost of goods affordable by applying fundamental knowledge of the biology of the cell and a sound understanding of engineering principles. Below we briefly discuss a few areas of challenges and opportunities.

1.5.2.1 Cell Isolation

Cell-based therapeutics must attain cells from a donor originally. An ideal process for many allogeneic applications would involve expanding the isolated cells, performing quality control, and then using these cells to generate master and working cell banks for manufacturing cell production. This production process would not require frequent cell isolation. It would reduce quality variability due to cell source but also allow for systematic process optimization to give robust performance. PSCs or other highly proliferative stem cells that are expanded in culture fall into this category. In other allogeneic applications and in autologous treatments, cells need to be routinely isolated from new patients. Means to increase the efficiency and yield of cell isolation from tissues depend on the tissue of interest but will be important for process robustness. Even for isolation from peripheral blood, not all cells are easily accessible. HSCs reside in their long-term or short-term niches and stay at a state that is a balance between self-renewal and differentiation. Long-term repopulating cells (stem and progenitor cells) are present in the endosteal niche, while short-term repopulating cells reside in the vascular niche [89]. HSCs used to be isolated from bone marrow. Nowadays, HSCs are mostly collected from peripheral blood. HSC mobilization from the localized bone marrow niche to the peripheral circulation in the blood increases after the patient is treated with various exogenous stimuli such as cytokines (G-CSF, GM-CSF), subjected to stressful conditions (i.e., injury or inflammation), and/or given myelosuppressive chemotherapy [29, 46].

1.5.2.2 Cell Expansion, Recovery, Formulation, and Delivery

In the current biomanufacturing of therapeutic proteins, each reactor run produces sufficient material for thousands of doses. Such economy of scale is not only pertinent to labor and the depreciation of capital investment but also affects the cost associated with quality evaluation of the product. In the future, cell manufacturing processes will likewise need to generate a very large number of doses per reactor run to be economically viable for most products.

Most cells of interest for therapy and regenerative medicine are adherent, except for those isolated from peripheral blood, such as T cells. They require a compatible surface to attach on for cell proliferation. The scale-up for the cultivation process of those adherent cells that are also required at high doses will be challenging.

For therapeutic applications, the cell product must be of high quality in terms of its intended biological activities, viability, and lack of contamination. In the production of therapeutic proteins, the product is purified from the cells, sterilized, and kept in a stabilizing formulation. For therapeutic cell applications, cells must be sustained in a highly viable and functional state and cannot be subjected to extensive purification and sterilization. They also need to be kept in a high-quality state for short-term distribution to clinic sites or even for a longer inventory period.

1.5.3 Cells and Biomaterials

Biomaterial strategies have been increasingly used for homogeneous expansion and differentiation of cells as well as for delivery of cells to the patient. The natural extracellular matrix (ECM) maintains tissue architecture while also acting as a reservoir for growth factors. The ECM possesses mechanical properties that support the required organ load while also supporting cell attachment and migration. In addition, the ECM often supports

specialized cell functions. Approaches using decellularized tissues as scaffolds for regenerative medicine have shown promise [72], and advances in imaging and 3-D printing have improved the ability to template and pattern scaffolds based on natural ECM.

Synthetic materials have also been widely explored, as they provide the ability to more closely tune the environment, are often more scalable, and do not exhibit as much batch-to-batch variability as natural ECM. The degradation of the synthetic material can be tuned, allowing controlled delivery of various cargo. In addition, stimuli-responsive materials with properties that are affected by light or temperature have enhanced both the delivery of the material to a desired site in the body and the in vivo tunability. Substrate stiffness, nanotopography, and chemical composition of synthetic materials have been shown to drive stem cell fate decisions in various contexts [50]. In addition, advances in microfluidic and miniaturized systems have also enabled exploration of a wide biomaterial parameter space in optimization of both bioreactor systems and scaffold functionality [56]. 3-D patterning methods have facilitated development of hybrid synthetic and natural ECM scaffolds that can mimic the complex environment of native tissues, including gradients in chemical signals [3].

1.6 Concluding Remarks

In the past century, scientists have expanded our capability to culture mammalian cells tremendously. The initial drive from scientific inquiry has led to a very large technological sector for the production of vaccines and therapeutic medicines. Mammalian cell culture now promises to become the next generation of medicine through regenerative therapies. The cells of interest have also extended from primary isolates of tissue to differentiated cells derived from tissues and stem cells. Nevertheless, the fundamentals of transforming cell culture practices in the laboratory into manufacturing process technology remain the same. We will likely see many innovations in the near future that harness the potential of the next generation of cell bioprocess technology.

Take-Home Message

- Cell culture has progressed from a simple research tool to a valuable method for the production of protein therapeutics.
- Stem cells and cell therapies are emerging as the next generation of medical technology.
- Innovations in biomanufacturing are still needed for cell therapies to be produced at a large scale.

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Essentials in Cell Culture

Antonina Lavrentieva

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What You Will Learn in This Chapter

When starting to work in mammalian cell culture, it is essential to understand the major principles of in vitro cell cultivation. These principles include knowledge of basic cell biology, working under aseptic conditions, choosing the right cell lines, culture vessels and equipment, as well as general acquaintance with the existing methods for cell state monitoring. Even before crossing the lab door for the first time, one should already be armed with basic knowledge about cell growth and the processes behind it, as well as be familiar with the techniques to influence it. It must be said beforehand that there are no absolutely standard/uniform cell culture labs around the world. Equipment, plastic ware, and cell types are used depending on the final application. This can range from the specified production of valuable biomolecules to in vitro testing and basic research. In this chapter, a short history of cell culture development will be presented first. Afterward, we will cover the different aspects of modern mammalian cell culture including the variety of cell culture applications, the basics of working under aseptic conditions, cultivation vessels, cell growth kinetics, and the evaluation of cell cultures.

2.1 Basics of In Vitro Cell Cultivation

2.1.1 Short History of Cell Culture: Pioneers and Milestones

As you have already learned in ▶ Chap. 1, animal cell culture has a history of over a 100 years. Scientists **Ross Harrison**, **Wilhelm Roux,** and **Leo Loeb** established the first tissue cultures at the end of the eighteenth and beginning of the twentieth century. In the first animal cultures, pieces of animal tissue were cultivated in a mixture of animal blood and warm saline or were implanted in animals as grafts of agar-encapsulated tissue [25]. Ross Harrison was also the first researcher to apply a reproducible technique called "hanging drop" for the cultivation of microtissues. Using this method, small pieces of tissue were cultivated in a drop of fluid (e.g., fresh lymph) on an inverted coverslip. Later in 1911, this method was successfully used by **Montrose Burrows** and **Alexis Carrel** to cultivate various embryonic and adult tissues in fresh plasma from the same animal source [5]. Furthermore, Burrows and Carrel used serum and artificial medium, cultivated tissues in hanging drops but also in watch glasses placed in glass plates, and were also the first researchers to cultivate small tumors in vitro [6]. Interestingly, Burrows and Carrel were also the first ones to isolate and cultivate tissues using "scaffolds" – in their case: pieces of silk or cotton [6]. In 1911 **Warren Lewis** studied and characterized the influence of factors responsible for the survival and growth of the cells in the culture medium [3]. In 1916 **Francis Peyton Rous** and **FS Jones** first introduced trypsin for the preparation of cell suspensions [20]. In 1921, in cooperation with **Albert Ebeling**, Carrel reported the successful long-term cultivation of fibroblasts derived from chicken embryonic heart – these cells could be cultivated for over 9 years [7]. Interestingly, even without the use of antibiotics (which were simply not discovered yet) and trypsin (Carrel and Ebeling subcultivated the cells by cutting out the growing part of the population), researchers were able to hold these fibroblasts incredibly long in culture – Ebeling took over these cells and cultivated them over 34 (!) years. According to Carrel's and Ebeling's theory, isolated animal cells could divide indefinitely in vitro, and cell aging took place only in vivo in multicellular organisms. However, such long-term cultivations could not be reproduced by other scientists, which later led to a paradigm shift in the understanding of cell life span limits [27].

Fig. 2.1 First T-flasks with a conical tip, developed by Wilton Earle in the 1940s. (Courtesy of the Office of NIH History and Stetten Museum)

The discovery of penicillin in the 1940s was the next important step in the development of cell culture, since the addition of antibiotics to the cell culture media significantly reduced undesired contaminations. Also in the 1940s, **Wilton Earle** at the National Cancer Institute designed flasks made from glass tubing ("T-flasks") with a conical tip (Fig. 2.1), developed chemically defined media for cell culture, and established the first continuous cell line, the *L-cell mouse fibroblasts*. In 1948 the first mouse cell strain was cloned, L929 cells, which are still widely used in labs all over the world. Another important milestone in the establishment of cell cultures was the development of a cell culture medium by **Harry Eagle** in 1955 [9], which was modified by **Renato Dulbecco** (the well-known Dulbecco's Modified Eagle Medium – DMEM). Renato Dulbecco also reintroduced the application of trypsin for cell detachment, which was forgotten after the first discovery for several decades.

Undoubtedly, one of the major milestones in mammalian cell culture development was the isolation of *HeLa cells* in 1951 by **George Gey**. Until that time, all attempts to grow human cells in vitro failed – and only cells from rodents were cultivated successfully. The history of **Henrietta Lacks** cells started in tragic circumstances – a 30-year-old woman went to the hospital because of nonperiodic bleedings and a biopsy of her cervix was taken for diagnosis, which revealed a malignant epidermoid carcinoma (later diagnosed as adenocarcinoma) of the cervix. The biopsy sample was used by George Gey to attempt to grow cells in vitro – and for the first time in history, it worked: the cells of Henrietta Lacks (HeLa cells) were dividing and growing without any limitations. Henrietta died that same year and never learned that her cells became the first immortal cell line, still widely used in laboratories all over the world. With the help of HeLa cells, it was possible to successfully propagate poliomyelitis viruses in vitro and to develop vaccine against poliomyelitis [22]. HeLa cells were used for numerous research topics including cancer, the influence of toxic substances, and radiation. The case of the HeLa cells also raised serious ethical discussions, since her family did not know for decades that Henrietta's cells were being used for research. This situation happened, because cell culture technology was developing much faster than ethical regulations.

As already mentioned in ▶ Chap. 1 of this book, in 1961 **Leonard Hayflick** and **Paul Moorhead** discovered that human primary cells in in vitro cultures can only divide a finite number of times [15]. The researchers studied the in vitro replicative capacity of primary human fibroblasts and made the conclusion that aging is happening on the cellular level (this was in conflict with existing knowledge at the time that cells in vitro can divide infinitely). This discovery changed the central dogma in cell aging, and the process of stopping cell division in vitro is now known as "the Hayflick's limit" (see also section "Cell Growth Kinetics" in this chapter) [23].

The isolation (in 1958) and further development (in the 1980s) of the *Chinese hamster ovary (CHO) cells* was the next milestone in mammalian cell culture technology. The major advantage of CHO cells is their ability to grow in chemically defined serum-free medium in suspension, which makes large-scale cultivation of these cells possible (scale-up here is performed by volume and not by growth area). Nowadays, these cells are one of the major host cell lines for the industrial production of recombinant proteins and antibodies.

One of the most interesting and still not fully studied mammalian cells are stem cells. These cells are not only able to proliferate (self-renewal) but can also differentiate into other cell types. *Embryonic stem cells (ESC)* were fist recognized to be a cell causing teratoma in animal experiments performed by **Kleinsmith and Pierce** already in 1964 [17], and in 1981 these pluripotent cells were isolated by **Evans and Kaufman** from the inner mass of very early mouse embryos [10]. *Mesenchymal stem cells (MSCs)*, often called also multipotent stromal cells, were first isolated in 1970 from guinea pig bone marrow aspirates by **Alexander Friedenstein** and later found in almost all postnatal tissues [12]. In 2006, **Shinya Yamanaka** and **Kazutoshi Takahashi** were able to turn differentiated adult cells into pluripotent stem cells for the first time. *Induced pluripotent stem cells (iPSCs)* were created by the reprogramming of embryonic cells and differentiated adult mouse fibroblasts [24]. In 2012 Yamanaka together with John Gurdon received the Nobel Prize in Physiology or Medicine for the discovery that mature cells can be reprogrammed to become pluripotent.

2.1.2 The Variety of Cell Culture Applications

There is a wide spectrum of processes where mammalian cell cultures find application. First of all, most *basic research* is performed in cell culture. Immortalized cell lines from almost all cells types are now available for in vitro studies. Such simplified "one-cell-type" systems are used to study the molecular mechanisms behind cellular processes, as well as for the better understanding of intercellular interactions.

Another field of mammalian cell culture application with significant economic importance is *biopharmaceutical manufacturing*. Biopharmaceuticals are defined as "medical drugs produced using biotechnology, that is, therapeutic products created through the genetic manipulation of living cells or organisms" [28]. Biopharmaceuticals are large molecules, nucleic acids, peptides, or proteins which are not extracted directly from the natural biological source and are not synthesized chemically (◘ Table 2.1) [28]. Some simple biopharmaceuticals can also be produced in prokaryotic cells (e.g., *E. coli*) or lower eukaryotes (e.g., yeasts or fungi). Complex molecules like glycosylated proteins can only be successfully produced using mammalian cells. If such proteins do not contain their specific glycosylation pattern, they lose bioactivity, become unstable, or display misfolding. The in vitro manufacturing of such molecules has made it possible to treat

Table 2.1 Fields of mammalian cell culture application

Field of application	Examples of application
Basic research	Study of cell physiology under different conditions, cellular mechanisms of disease/disorders, drug development, cell-cell and cell-substrate interactions
Biopharmaceutical manufacturing	Production of: Proteins: cytokines, erythropoietin, insulin, interferon, interleukins, etc. Enzymes: trypsin, imiglucerase, Factor IX Nucleic acids: DNA, RNA, plasmids, etc. Monoclonal antibodies Vaccines
Biocompatibility and drug testing	In vitro biocompatibility evaluation of medical products (implants, catheters, stents, etc.) and nanoparticles (e.g., from sunscreens) Drug testing: High-throughput screening of anticancer molecules Inhibitors Bioactive molecules Drug delivery systems
Personalized systems	Cultivation and study of isolated tumor tissues/cells from patient
Cell-based therapies	Chimeric antigen receptor (CAR) T-cell therapy Treatment with MSCs
Tissue engineering	Bone tissue engineering Cartilage constructs Engineered vascular grafts

a variety of disorders without the risk of disease transfer. For example, cadaver-sourced growth hormone was shown to be associated with the risk of prion infection [1]. The amount of in vitro manufactured monoclonal antibodies has grown exponentially over the last few years. Such antibodies play an important role in the treatment of cancer and autoimmune and inflammatory diseases [28]. The production of vaccines saves millions of lives each year.

Biocompatibility and drug testing is another important field of mammalian culture. All new materials which are intended for human use (or may come in contact with humans) must undergo a complex biocompatibility evaluation (substance validation). This means that before new substances are tested in animal models or used in humans, they are first studied in vitro with cell cultures. In vitro toxicity evaluation is a relatively fast and simple method. There are certain guidelines in Europe, the USA, and worldwide which describe the procedures and requirements for such testing. The use of cell cultures also reduces the amount of animal experiments, since the obviously toxic materials can be excluded from further testing. Drug screening in cell culture allows the identification of the most active substance from a series of drug candidates. It also helps to define the exact mechanism of drug action and detects possible undesirable side effects on the cellular level. Traditionally, drug screening is performed on monolayer two-dimensional cell cultures. New developments of such screening methods use three-dimensional cell culture systems, which come closer to the real in vivo cell microenvironment (e.g., in the modeling of tumor tissue).

We will take a closer look at various 3D cell culture techniques and their advantages in ▶ Chap. 5 of this book.

A relatively new development in drug screening are *personalized systems*, where cells (e.g., tumor biopsy) are obtained from the individual patient and cultured in vitro in order to study the potential efficacy of anticancer drugs. Such systems represent cell heterogeneity, diversity, and sensitivity of tumors much better than immortalized cancer cell lines and provide more information about treatment efficacy and prognosis. Another example of personalized systems is the in vitro cultivation and study of primary cells isolated from patients with specific diseases in order to understand the pathological mechanisms underlying these disorders.

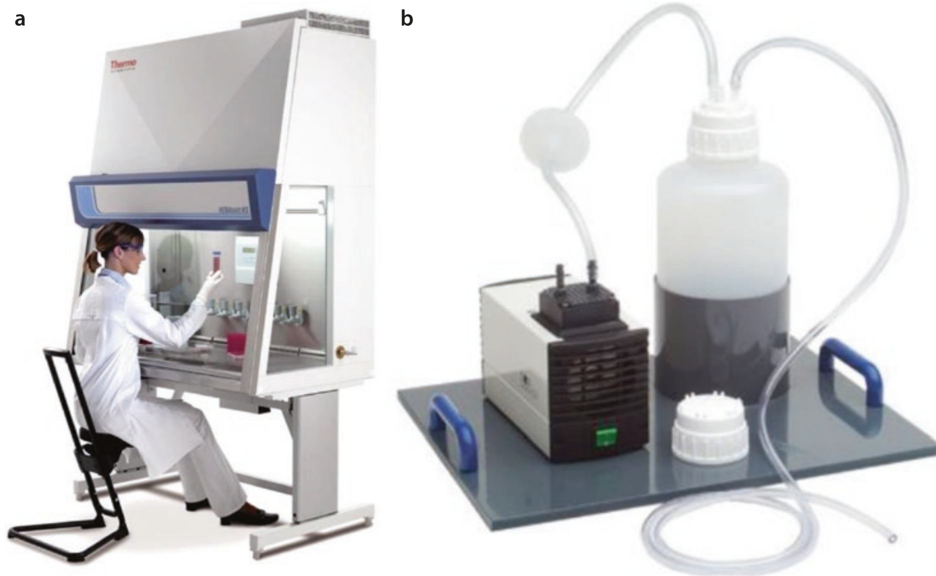
Tissue engineering and cell therapy are relatively new fields of cell culture application. In the case of cell therapy, cells are usually isolated from human blood, bone marrow, or tissues, manipulated in vitro (e.g., expanded, sorted, or genetically treated/manipulated), and transplanted into the same person where they were taken from (autologous transplantation) or to another person (allogenic transplantation). Typical examples of cell therapies are the transplantation of mesenchymal stem cells (MSCs), T-cell therapy, or chimeric antigen receptor (CAR) T-cell therapy. At the moment, there are 14 products approved by the FDA from the Office of Cellular, Tissue, and Gene Therapies (OCTGT) [11]. Moreover, thousands of cell therapy clinical trials are currently running; among others there are 686 trials for MSCs therapy alone and 4.629 – for T-cell and CAR T-cell therapy (▶ clinicaltrials.gov).

During cell therapy, cells are injected into the patient in the form of a cell suspension, while in tissue engineering, special tissue-specific artificial or natural constructs (scaffolds) are seeded with cells, cultivated in vitro (under static conditions or in special bioreactors), and subsequently implanted into the patient. For the successful production of the construct, the interaction of three major parameters is required: scaffold, cells, and cultivation conditions (medium composition, mechanical stimulation, oxygen concentrations, bioactive molecules, etc.). The importance of the abovementioned factors is explained further in ▶ Chap. 3 (cell culture media), ▶ Chap. 6 (biomaterials), ▶ Chap. 7 (cell surface interactions), and ▶ Chap. 9 (mimicking physiology in cell culture). One of the still existing problems of tissue-engineered constructs is their full vascularization in the body and stability/biodegradability. The most intensively studied directions in tissue engineering are artificial bone constructs, cartilage constructs, and vascular grafts production.

2.2 Work under Aseptic Conditions

2.2.1 Cell Culture Hoods

A common requirement of all cell culture labs in the world is working under aseptic conditions. Successful work in cell culture, as well as obtaining reliable and reproducible results, depends on the full control of undesired contaminations. In order to avoid contamination from airborne particles, all manipulations with cells are performed in a cell culture hood (biosafety cabinet) – a closed workspace where the air is constantly filtered (◘ Fig. 2.2a). There are three classes of cell culture hoods: class I (provide protection of personnel and environment and work in a manner similar to chemical fume hoods but are less effective in the protection of cells from contaminations), class II (provide both personnel and cell culture protection and can be used for potentially pathogenic organisms and hazardous



◘ **Fig. 2.2** **a** An example of cell culture hood of class II (Thermo Scientific Laboratory Products, Germany), **b** a vacuum pump system (Köhler Technische Produkte, Germany)

materials in biological safety levels up to 3), and class III (provide the highest protection level for personnel and are used in biological safety levels up to 4). Before and after work, the hood must be cleaned with disinfection solution (e.g., 70% ethanol or isopropanol).

It is important to keep only materials currently required for an experimental step under the hood and not to clutter the working space or use the hood for general storage. Most hoods have integrated UV lamps, which can be used at the end of the day for sterilization (minimum 30 min on). Remember, only uncovered/free surfaces are disinfected under UV light, which means that hoods must contain only a minimum of utilities/bottles. The standard set of utilities in the hood consists of pipettor for serological pipettes, tube racks, a waste jar, and sterile pipette tips. Optionally, a vacuum pump system can be used in the hood (◘ Fig. 2.2b). When you start working with your cells, you may bring the following items into the hood: buffers, media and reagents, cell culture flasks, sterile tubes, and serological pipettes. Before placing your materials into the hood, you can additionally disinfect them with 70% ethanol or isopropanol. The latest guidelines, however, recommend not using chemical disinfection solutions (at least not as aerosols), since chronic exposure may bring harm to personnel.

2.2.2 Personal Hygiene

The major source of cell culture contaminations (see section “Contamination” in this chapter) is the cultivator herself. The human clothes, skin, hair, and aerosols released from the nose and mouth may contain bacteria, mycoplasmas, and spores of fungi/molds. This is the reason why it is important to wash and disinfect your hands before and after work with cell cultures. Gloves, lab coat, and lab shoes must be worn during the entire stay in the cell culture lab – they will protect the cells from contamination coming from you and will protect you from possible hazardous materials you may be working with. It is

desirable to refrain from talking or singing while working in the hood. If you have long hair, it is a good idea to tie it back together. If you are already wearing gloves – do not touch your hair and skin. If the sleeves of the lab coat are too short, sleeve protectors can be worn additionally. Working with cell culture supplements of animal or human origin brings additional risk of infection in the case of accidental penetration in the organism (via pick with a needle or an accidental cut with the scalpel) – additional vaccination against hepatitis B may be reasonable before starting to work with human materials.

2.2.3 Sterilization Methods

There is no universal sterilization method for the materials and equipment used in cell culture: the appropriate technique must be chosen depending on the nature and properties of the materials to be sterilized. The chosen sterilization method should be compatible with the material in order to avoid its damage. Generally, sterilization is defined as the complete removal/elimination of bacteria, viruses, fungi, and spores from the (possibly) contaminated objects/materials. The major ways of sterilization of materials used in cell culture are listed below.

Steam sterilization is performed in almost every cell culture lab in the world. With this method, microorganisms (and their spores) are eliminated by heating the materials in saturated steam under pressure with the help of an autoclave. The minimum exposure required by steam sterilization is 121 °C and 30 min. The main disadvantage of this method is that application is possible only for heat resistant materials (e.g., glassware, metal, Teflon). Moreover, some delayed effects on materials, like corrosion and altered elasticity/transparency, can appear after steam sterilization. The advantage of steam sterilization is that it is nontoxic and relatively inexpensive. Color-changing adhesive autoclave tape (Bowie-Dick tape) is usually used to confirm if the desired temperature was reached. This tape is placed on the items before autoclaving and changes the stripes color from light beige to brown-black as temperature reaches 121 °C. It is important to note that the change of color is not dependent on the duration of steam sterilization and therefore cannot fully ensure that the material is sterile. Biological indicators (ampoules with bacterial spores) can be used to ensure successful sterilization.

Irradiation (gamma irradiation and UV exposure): with this method contaminants are killed by ionizing (gamma) or non-ionizing (UV) radiation. Gamma irradiation is the major method of cell culture plastic sterilization used in industry. In order to avoid induced radioactivity, the use of sterilization radiation higher than 5 MeV is prohibited [2]. Commercial gamma-ray irradiation facilities utilize the radioactive decay of cobalt-60 (^{60}Co) or cesium-137(^{137}Cs) and use the highly penetrating radiation, which travels nearly with the speed of light, to kill microorganisms in already packaged products. Irradiated products remain sterile until the packaging is removed [2]. Gamma irradiation irreparably damages life-sustaining molecules of microorganisms (e.g., DNA) and is used for the sterilization of plastic, instruments, devices, and even liquids (trypsin and serum). The usage of gamma irradiation for serum sterilization is however under discussion since free radicals produced during irradiation destroy proteins and other important molecules in serum charges even during sterilization by low temperatures. Main advantages of gamma sterilization are high penetration, better assurance of product sterility, and the absence of toxic residuals. The presence of oxygen, the absence of water, and a slightly elevated temperature increase the efficiency of gamma sterilization. The resistance of a microorganism

is measured by the decimal reduction dose (D_{10} value), which is defined as the radiation dose (kGy) required to kill 90% of the irradiated microorganisms. The irradiation dose for sterilization is dependent on the resistance of the organisms to be eliminated: in general, it is known that yeasts and spores are more resistant to gamma irradiation than bacteria; viruses are more resistant than spores, molds, yeasts, and bacteria; and double-stranded DNA viruses are more resistant than single-stranded DNA viruses. The major disadvantage of gamma sterilization is the usage of radioactive materials with consequently strictly regulated equipment installation, usage, and disposal.

UV irradiation is inexpensive and less dangerous than gamma irradiation. However, it displays low penetration and lower efficacy, needs longer time, and can also affect materials during sterilization. In this method, short-wavelength ultraviolet (UV-C, 200–270 nm, treatment time at least 30 min) is used to eliminate microorganisms by breaking the molecular bonds in DNA and the formation of thymine-thymine dimers. Some bacteria, however, have DNA-repairing mechanisms, which makes UV irradiation noneffective against such contaminants. Most modern biosafety cabinets have built-in UV lamps that are used for sterilization/disinfection of inner surfaces after work.

Sterile filtration: all heat-labile liquids (culture media, antibiotics, amino acids, etc.) which cannot be autoclaved must be sterilized by membrane filtration. There are several types of such filters available on the market – from small syringe filters to big bottle top filters, the pore size varies from 0.1 µm to 0.5 µm (Fig. 2.3). Filtration is performed either by positive or by negative pressure created by peristaltic and vacuum pumps or syringes. The membranes used in filters must not adsorb proteins, peptides, and other important molecules from the filtered fluids. It is important to note that mycoplasmas and viruses cannot be removed by such filtration, although 0.1 µm pore size is more effective for the removal of mycoplasmas from solutions. This is why it is essential to regularly check the running cell cultures on the presence of these contaminants (see section "Contaminations" in this chapter). Air in the biosafety cabinets is filtered with high-efficiency particle air (HEPA) filters, which remove nearly all particles lager than 0.3 µm.

Chemical sterilization methods can be divided depending on their (I) state: to liquid (with alcohols, aldehydes, and halogens) and gaseous (with ethylene oxide or formaldehyde) sterilization and (II) mechanism of action, protein denaturation (alcohols,

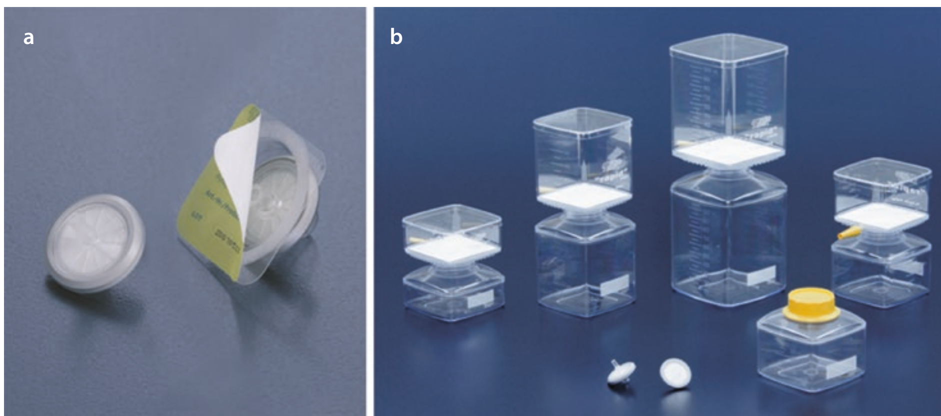


Fig. 2.3 **a** Syringe filters and **b** bottle top filters for sterile filtration in the cell culture (TPP Techno Plastic Products AG, Switzerland)

aldehydes), action on membranes (detergents, alcohol), and damage of nucleic acids (formaldehyde, ethylene oxide). The major gaseous disinfectant, ethylene oxide, is used for sterilization of thermolabile materials and can effectively eliminate microorganisms and spores. It is however highly flammable and usually used as mixture with CO_2 (90% EO and 10% CO_2). Moreover, it is highly toxic, and residuals can affect cell growth after sterilization. Liquid sterilization with alcohols is usually performed with 70% ethanol or 70% isopropanol. With this concentration, cellular dehydration, protein coagulation, and membrane disruption of microorganisms take place. It is important to use exactly 70% solutions since higher or lower concentrations are less effective in the elimination of contamination. In the past, aldehydes (e.g., 40% formaldehyde aqueous solution) were also used for disinfection, as they are effective for the inactivation of microorganisms by alkylating the amino and sulfhydryl groups of proteins and ring nitrogen atoms of purine bases. In modern cell culture labs, aldehydes are not preferable as disinfectants, since long-term exposure to low levels in the air or on the skin can cause asthma-like respiratory problems and skin irritation. Moreover, formaldehyde is considered to be carcinogenic, and researches/technicians should limit direct contact with this chemical. These considerations limit the role of aldehydes in sterilization and disinfection processes. When using chemical disinfection, make sure that you treated all surfaces of the material you want to disinfect. Chemical disinfectants are also toxic for the cells you are cultivating – make sure that your cells do not come in contact with the aerosol/evaporant.

2.2.4 Contaminations

Contaminations represent the major problem of all cell culture techniques. They cause irreversible damage of running experiments in basic research and make produced biopharmaceuticals in industry unusable. If contaminated, cultivated cells must be discarded in most cases, since there are no existing reliable methods to fully eliminate the contamination without affecting the cells. Contaminations cause loss of labor, time, and money. Despite the use of antibiotics in the media, resistant bacteria can still grow in the cell culture. Moreover, invisible/undetectable (with a microscope) or suppressed (by use of antibiotics) contaminations affect cell viability and reproducibility of the experiments. Major cell culture contaminations are caused by *bacteria*, *fungi*, *mycoplasmas*, and *viruses*. If the first two are easily detectable under a microscope, smaller organisms can contaminate the culture without being detected over long periods of time. Finally, another contamination type is the *cross-contamination* with other cell types. All these biological contaminations will be described and discussed in this section.

Bacterial contamination is the most common and frequently occurring cell culture contamination. Even after the discovery of antibiotics, bacterial contaminations remain a constant and undesirable satellite of all cultivated cells. Bacterial contamination can be easily detected by microscopic changes in medium color (pH shift) and turbidity. Under the microscope, bacteria can be seen as moving or non-moving particles (dots or rods) in the micrometer size range in the space between cells (◘ Fig. 2.4). Sometimes it is difficult to distinguish between cell culture medium precipitates (e.g., proteins from serum or cell debris) and bacteria, but high magnification microscopy can help to reveal bacteria. It is important to note that small nonbacterial particles can also move – they show Brownian motion. Another possibility to differentiate precipitates from bacterial contamination is to take a supernatant sample, place it in a separate vessel without cells, and monitor the

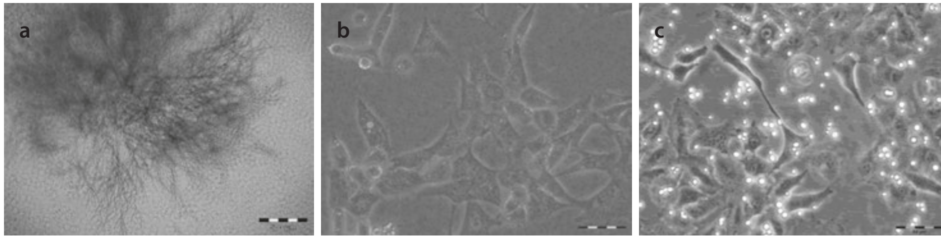


Fig. 2.4 Examples of molds **a**, bacterial **b**, and yeast **c** contamination in cell culture

glucose/lactate concentration over several days: if the glucose concentration decreases – most likely a bacterial contamination has occurred. The major source of bacterial contamination in cell culture is the water bath, which is why it is recommended to regularly empty and clean it. Another warm and humid environment favorable for bacterial growth is provided by cell culture incubators – here again regular decontamination via cleaning with chemical disinfectants is recommended. Moreover, some incubators have additional decontamination function. In this equipment, the whole incubator chamber is heated up to 100 °C in a humid atmosphere for several hours. Copper walls and shelves in incubators play additional bactericidal role. While working in a biosafety cabinet, it is recommended to refrain from touching pipette tips, pouring fluids from bottles into flasks, and accidentally contacting surfaces with sterile pipette tips.

Fungi contamination, the second most common biological cell culture contamination, can also be easily detected by medium turbidity (yeast contamination) or floating furry clumps of mycelium (molds). Under the microscope, yeasts appear as asymmetric pearls or chains of pearls and molds usually as multicellular filaments (Fig. 2.4). Normally, during early fungus contamination, the pH of the cell culture medium stays unchanged, and later it increases. Cell cultures are usually contaminated with fungus via an airborne route and contact with contaminated materials.

Bacterial and fungus contaminations are easily detectable under the microscope. Mycoplasma contamination, on the other hand, can remain undetected for a long time and can influence the outcome of experiments and biopharmaceutical production.

Mycoplasmas are small self-renewing prokaryotes without cell walls about 100 nm in diameter. The lack of cell walls makes them resistant to antibiotics acting on cell wall synthesis, as well as to lysozyme. During contamination, mycoplasmas enter the host cells and rapidly proliferate, influencing host cell physiology. Moreover, mycoplasmas compete with the cell culture for nutrients and important molecules, leading to decrease in cell growth and production of ATP and proteins. Mycoplasmas contaminations do not cause medium turbidity and do not build films on the cell culture flasks bottom. The major source of mycoplasmas contamination is other cultivated cells or primary tissues, the major route of contamination – aerosols, medium residuals, and reused pipettes. Every cell culture must be regularly tested on the presence of mycoplasmas. Newly arrived cells in the lab must first be cultivated separately to other cells until the absence of mycoplasmas is confirmed. Another source of mycoplasmas contamination is serum and other products of human or animal origin. Laboratory personnel can also carry mycoplasmas and be a contamination source. The main tests to detect mycoplasmas include PCR kits (e.g., with primers for 16S rRNA coding region in the mycoplasmas genome), cell-based tests (e.g., based on the ability of mycoplasmas to activate the immune cell response), and staining with DAPI or Hoechst dye (to detect mycoplasmas DNA in the host cell cytoplasm).

Viruses represent another serious threat to cell cultures since they are also difficult to detect and almost not possible to eliminate. Moreover, virus-contaminated cells are dangerous for personnel since they can be highly pathogenic. Some viruses cause changes in cell morphology which indirectly may indicate contamination, but some viruses remain undetected. Similarly to mycoplasmas, virus contamination originates from contaminated cell lines (e.g., if the animal or human from which cells were isolated was infected) and untreated animal or human materials. Cells contaminated with viruses cannot be disinfected and must be discarded.

Although *cross-contaminations* are not typically listed as contamination candidates, serious scientific and economical damage can be caused by them. Cross-contaminations are caused by contamination of the cell culture by other cells (e.g., feeder cells or other cell lines cultivated in the same lab/incubator). Here, we will show some examples of cross-contaminations which had a serious impact on the scientific society.

Isolation of the abovementioned HeLa cells was a breakthrough in human cell culture – these cells could be cultivated infinitely and grew very fast in vitro. Directly after isolation, HeLa cells were sent by George Gey to researchers in different institutes around the world. In the early 1960s, however, it became evident that HeLa started to contaminate other cell lines [18]. By 1967, Stanley Gartler reported the cross-contamination of 19 cell lines detected with the help of isoenzyme analysis of glucose-6-phosphate dehydrogenase (G6PD) and phosphoglucomutase (PGM) electrophoretic polymorphisms [18]. He revealed that all these cell lines have the same types of G6PD and PGM, which are mostly distributed in African Americans. It was unlikely that all tested cell lines were occasionally coming from this part of the population. Later, other methods including karyotyping and DNA barcoding confirmed the contamination of various cell lines with HeLa cells. Moreover, the WISH cell line, isolated by the famous scientist Leonard Hayflick (section "Cell Growth Kinetics" of this chapter) from his daughter's amnion, was also identified by Gartler as carrying the abovenamed genetic marker typical for African Americans. Since both Hayflick and his wife were white, it was unlikely that they initially carried this phenotype. It was reported that HeLa contamination appeared in the labs and cell banks all around the world, and this contamination could even have happened via air droplets [18]. By the time of cross-contamination identification, thousands of studies using these cell lines were already published. Besides the generation of incorrect scientific data and wasted years of research, HeLa contamination had a great impact on the development of anticancer treatments, since cell lines believed to be a certain cancer type were in reality all HeLa cells.

Another example of cross-contamination comes from the stem cell research area. In 2009, a scientific group from Norway published alarming results of frequent spontaneous malignant transformation of human BM-MSC in vitro [19], although another working group did not reveal any transformation of these cells 2 years earlier [4]. Rubio and colleagues also reported spontaneous transformation of AD-MSC in vitro, indicating the importance of biosafety studies of MSC biology to efficiently exploit their full clinical therapeutic potential [21]. In the year 2010, however, after a publication of the "identity crisis" editorial letter in Nature [16], where the misidentification of a tremendous amount of cell lines used in laboratories around the world was discussed, numerous working groups decided to carefully identify the origin of the cells they were working with. The results of the DNA fingerprint analysis revealed that the above-reported malignant transformation of BM-MSC was reflecting cross-contamination with the human HT1080 fibrosarcoma, U251 and U373 glioma, and U-2 OS osteosarcoma cell lines [26] in two independent laboratories. The working group which reported spontaneous transformation of AD-MSC

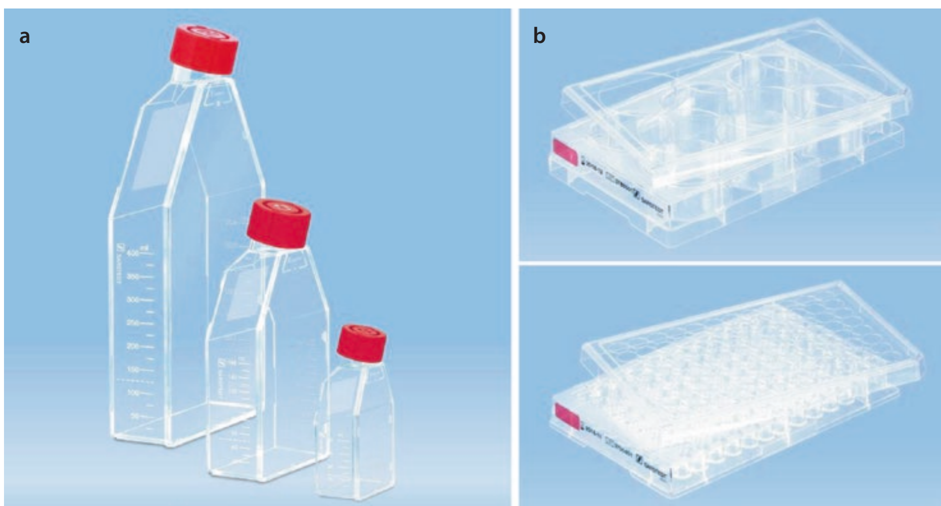
also identified cross-contamination of MSC with a HT1080 cell line [13]. Although these findings provide support for future MSC applications in patients, they also demonstrate the importance of safety regulations during in vitro expansion of these cells, in particular with regard to cross-contaminations.

2.3 Cultivation Vessels

2.3.1 Simple Cell Culture Flasks, Culture Flasks

Most adherent mammalian cells are cultivated in the so-called T-flasks since the development of this kind of vessel in the 1940s (◘ Fig. 2.1). The first cell culture flasks were made of glass. However, primary cells have difficulties attaching to glass surfaces, and researches were trying to use various proteins to improve cell attachment. Modern cell culture flasks and microplates are disposable, single-use vessels which are mainly made of *polystyrene* because of its good optical properties, easy casting during manufacturing, acceptable costs, and the possibility of surface modification. Since polystyrene is hydrophobic, all cell culture vessels made of it are treated with gas plasma or corona discharge in order to oxidize it, thus rendering the material hydrophilic and negatively charged. Moreover, surfaces can be modified with amino (NH_3^+-), carboxyl (COO-), and other functional groups. It is important to remember that different cells attach differently on various surfaces. For some cell types, oxidized polystyrene is sufficient for good adhesion; for others special surface modifications and coatings are required. In particular, cells cultivated in serum-free medium frequently require special surface coatings for attachment. Surface coatings can be made with extracellular matrix proteins (e.g., fibronectin, collagen, or laminin), with synthetic polymers (e.g., poly-D-lysine), or with mucopolysaccharides (e.g., hyaluronidase and heparin sulfate).

Cell culture flasks for adherent cells have different sizes. The most widely distributed types are T25 (25 cm^2 surface area), T75 (75 cm^2 surface area), and T175 (175 cm^2 surface area) (◘ Fig. 2.5). Knowledge of the exact growth area is important for seeding the



◘ **Fig. 2.5** **a** Typical cell culture flasks for adherent mammalian cell culture: T175, T75, and T25 flasks. **b** 6-well and 96-well cell culture plates (Sarstedt AG & Co, Germany)

required cell density, as well for estimating the average cell yield obtained. Multiwell plate sizes vary between 6-well plates and 386-well plates. Multiwell plates with high well numbers are usually used for high-throughput assays.

For the culture of suspension cells, no special treatment of surfaces for better attachment is required. Moreover, to keep cells in suspension, ultralow attachment surfaces are created (e.g., by coating with inert neutral hydrophilic hydrogels or polydimethylsiloxane). This treatment prevents proteins from the cell culture medium absorbing on the surface and hinders consequent cell attachment.

It is important to note that all in vitro static cultivations are performed in incubators (◘ Fig. 2.6), where T-flasks or multiwell plates are placed. Incubators provide optimal temperature (usually 37 °C), oxygen concentration (depending on the cell type), and humidity (95%) which prevents medium evaporation following osmotic stress, as well as CO_2 supply (5%) for pH maintenance with bicarbonate buffer. High humidity in incubators is usually achieved by direct water fill into the incubator bottom, either by filling into a special tub placed on the bottom or by using active steam injection (steam generators). By their mode of temperature regulation, incubators can be divided into direct-heated and water-jacketed. The inner surfaces of incubators can be made of stainless steel (which is smooth and easy to clean) or copper (rough and difficult to clean but bactericidal).

Simple cell culture vessels, however, cannot really provide physiologically relevant cultivation conditions. Here, cells grow on the plastic surface in monolayers and under static culture conditions. Medium is being frequently manually replaced, thus withdrawing cell-signaling molecules. Moreover, because of the limited surface area available, only

◘ **Fig. 2.6** Incubators for mammalian cell cultures (Thermo Scientific Laboratory Products, Germany)

moderate cell amounts can be produced. Therefore, if the production of cells is to be scaled-up, the number of T-flasks has to be increased, making the cultivation of cells time-consuming and prone to contaminations. In order to increase the growth area, so-called *cell factories* were developed, where ten or more chambers are arranged in multilayer stacks. Cultivation in cell factories, however, still remains a static method with additional limits in cell growth monitoring. The latest promising development in in vitro cell cultivation is the application of diverse *bioreactors*. Here, various sensors can be integrated for control and documentation of important cultivation parameters like nutrients, gas and metabolites concentrations, pH, temperature, pressure, shear forces, and cell growth.

2.3.2 Bioreactors

Based on general setup and configuration, the bioreactor systems (shown in Fig. 2.7) can be divided into the following categories: *static*, *wave-mixed*, *stirred*, and *perfusion bioreactors*. The main difference between these concepts is associated with mass transport (e.g., diffusion, perfusion, or bubbling) and the suitability for adherent or suspension cell cultures. Wave-mixed and stirred systems can be used for suspension cultures, but with the aid of microcarriers, also adherent cell types can be cultivated in these systems. On the other hand, hollow fiber bioreactors are specially designed for the cultivation of adherent cells.

As already mentioned, the T-flask is the simplest vessel for adherent cell cultivation, where the inner surface on the bottom of the flask is used to culture cells in monolayers. Another system for adherent cell cultivation are the *hollow fiber bioreactors* (HFB) which

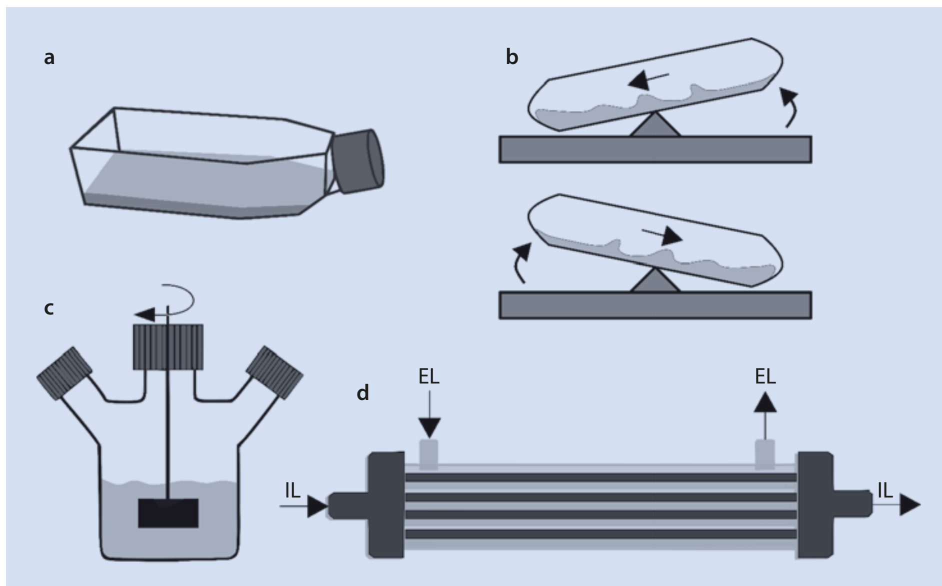


Fig. 2.7 Common systems in cell culture: **a** T-flask for static cultures, **b** wave-mixed bioreactors, **c** spinner flasks, and **d** perfused hollow fiber bioreactors with an intraluminal (IL) and the extra-luminal (EL) space

consist of fibers fixed into a module which offers a high surface area for cells to adhere and a continuous flow of media which is delivered through the fiber lumen. In Fig. 2.1d the schematic concept of a HFB is shown. In principle, the bioreactor consists of two spaces: the intraluminal (IL) and the extra-luminal (EL) space. This setup offers a physiological environment with the fibers mimicking blood capillaries.

For suspension cultures, stirred systems such as *spinner flasks* are usually used, which consist of a vessel and an impeller (see Fig. 2.7c). The impeller is used to mix the culture medium in order to provide a homogenous environment regarding nutrient supply, oxygen concentration, cell concentration, and temperature. Spinner flasks are a very flexible and scalable platform, since they exist in various forms, materials, and sizes (3–2000 l in volume). An even simpler form of dynamic cell cultivation can be achieved by placing a culture vessel on a shaker platform. Modern bioreactors using this principle are called *wave-mixed bioreactors*. A schematic concept is depicted in Fig. 2.7b, where a single-use plastic bag filled with culture medium and cell inoculum is placed on a motorized platform. The platform performs a rocking motion, which provides excellent mixing and gas transfer without damaging the cells in suspension.

2.4 Cell Growth Kinetics

2.4.1 Population Doubling, Cell Cycle

Mammalian cells (primary cells and cell lines) grow in culture attached to a surface or in suspension. Attached cells are also called anchorage-dependent and suspension cells, accordingly, anchorage independent. In vitro growing cells must be subcultivated in order to enable continuous growth. Cells divide (replicate) at different rates, and the time of subcultivation is different for various types of cells (see time-lapse videos using the QR-code in Fig. 2.12). Primary cells grow slowly and are usually subcultured at a 1:2 ratio, while the fast-growing immortalized cell lines need higher ratios. Each subculturing into a new vessel counts as a passage: for suspension cells it is the simple splitting (dilution) of the culture and for adherent cells, the detachment from the substrate and from other cells with the aid of proteases (in order to generate a single-cell suspension) followed by dilution. In both, suspension and adherent cell cultures, cell counting is performed before reseeding (see section "Estimation of Cell Number and Viability" in this chapter).

Population growth in mammalian cell culture can be described by means of *population doublings* and *population doubling time* (the time cells require to double in number). Population doublings are calculated using the following formula:

$$N_{\mathrm{d}} = \frac{\ln\left(\frac{X}{X_0}\right)}{\ln 2}$$

where N_{d} is the number of population doublings during a Δt period of time, X_0 is the number of living cells at time $t = 0$, and X is the number of living cells at time t. Population doubling time T_{d} is calculated with the following formula:

$$T_{\mathrm{d}} = \frac{\Delta t}{\mathrm{Nd}}$$

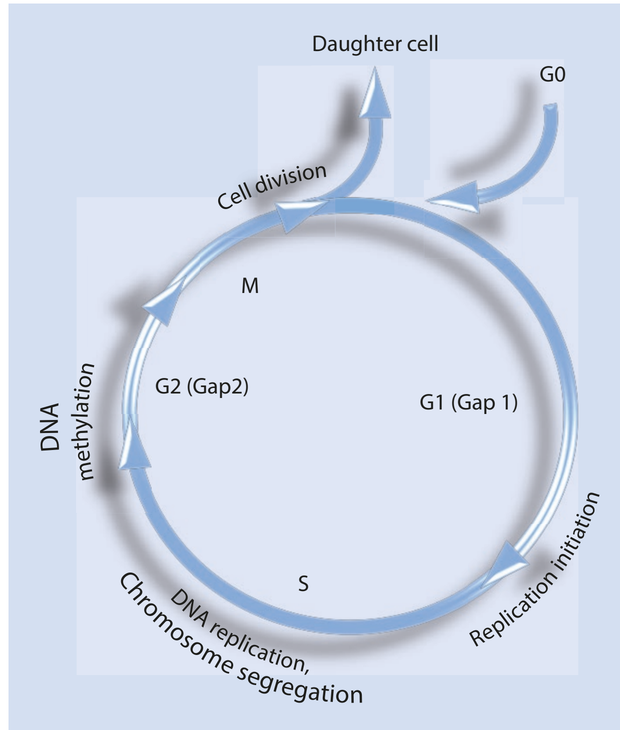


Fig. 2.8 Mammalian cell cycle

Doubling time in mammalian cell culture varies between 12 and 40 h depending on the cell type, cell culture medium, and added supplements. Cell replication is controlled by various factors and pathways, including extracellular signals and intracellular clues. In general, mammalian cell cycle can be divided into four discrete phases: *M phase* (mitosis), *G1 phase* (gap 1), *S phase* (synthesis), and *G2 phase* (gap 2) (Fig. 2.8). The most important part of cell division happens in the M phase: nuclear division. During the two gap phases, cells grow in size, proteins are synthesized, and cell cycle checkpoints are passed to control if the cell is allowed to enter the next phase. There are three major checkpoints in the entire cell cycle at which the cell cycle can be arrested: at the end of G1 phase (if DNA is damaged), at the end of G2 phase (if DNA is unreplicated or damaged), and at the end of M phase (in the case of chromosome misalignment). DNA replication occurs in the S phase. If the cell undergoes differentiation, in the case of contact inhibition or nutrient deficiency, it enters *G0 state* (G0-arrested cell).

One of the major features of mammalian cell culture (for both adherent and suspension cells) is the cell growth kinetics, which can be divided into four major phases: lag phase, log (exponential growth) phase, stationary phase, and decline (death) phase (Fig. 2.9). *Lag phase* starts directly after cell seeding in the new culture vessel (subcultivation). In this phase, cells grow very slowly, recovering after subcultivation and adapting to the new environment. Adherent cells need time for adhesion, reconstruction of the cytoskeleton, and the production of extracellular matrix proteins, which were partially digested by proteases during subcultivation. The protease treatment, which is used for detachment of cells from the cell culture surface, results in loss of surface proteins, attachment proteins, and receptors. During lag phase these proteins are resynthesized. Suspension cells adapt to the

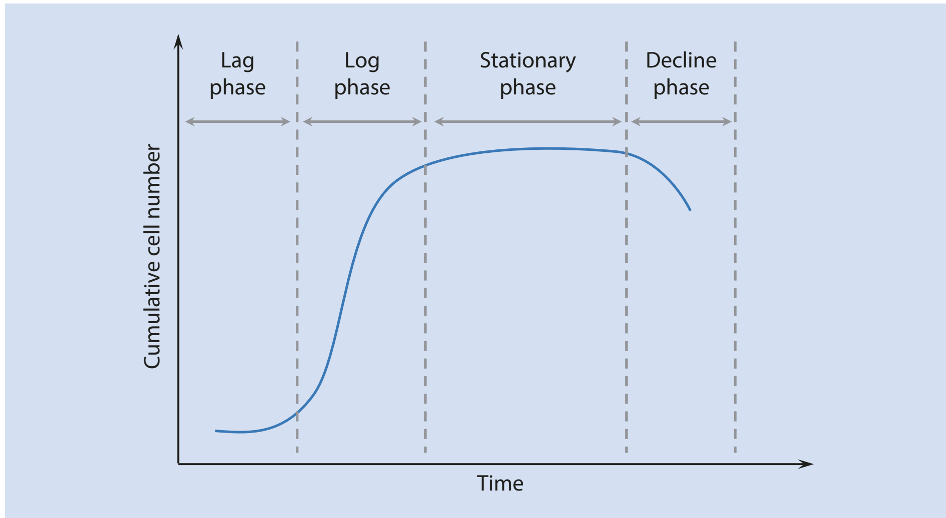


Fig. 2.9 Cell growth kinetics in a batch culture

temperature and the changed concentration of nutrients and signaling molecules. In *exponential (log) phase*, the cells divide at constant rate. This constant rate is dependent on the cell type, pH, temperature, oxygen, and nutrients. In the *stationary phase* cell proliferation slows down and stops. That is caused by high population density in suspension cultures and by contact inhibition in adherent cultures. In the *decline phase*, if the cell number is not reduced, cell viability decreases leading to the decrease of viable cell number.

As already mentioned previously, at the beginning of cell culture research, scientists believed that any cell placed into in vitro cell culture can divide infinitely (become immortal) and that cell mortality is only a property of multicellular organization. In 1961 Leonard Hayflick and Paul Moorhead published their study on human embryonic cells, where they showed that these cells double in vitro for only a finite number of times. After this limit has been reached, the cells stopped dividing and entered "the phase III" phenomenon. In contrast to immortal cancer cells, normal human cells divided in phase I (primary culture) and phase II (robust cell division), but in the third phase, replication stopped. Moreover, cells could "remember" how many times they have replicated before cryopreservation and after thawing divided only for a certain number of times. This "accelerated aging under glass" was later named the *Hayflick limit*, and its mechanism was gradually disclosed. First, the "replicometer" was located in the cell nucleus, and then it was discovered that it is the telomeres (noncoding ends of a DNA molecule with TTAGGG repeats) in the nucleus which are involved in the Hayflick limit and, finally, that it is actually telomeres shortening (attrition) which limits cell growth in culture. Telomere shortening causes *cellular senescence* – an irreversible growth arrest, where cells stop dividing but stay metabolically active. The main characteristics of senescent cells are the presence of several nuclei, large cell size, expression of β-galactosidase, and conversion to an immunogenic phenotype which in vivo makes it possible to eliminate these cells by the organism's own immune system. Cellular senescence is thus an important tumor-suppressive mechanism. The reason why some immortal cell lines and human tumor cells can divide infinitely is the presence of a special enzyme called telomerase, which causes telomere elongation. It is important to note that cellular senescence can be caused by

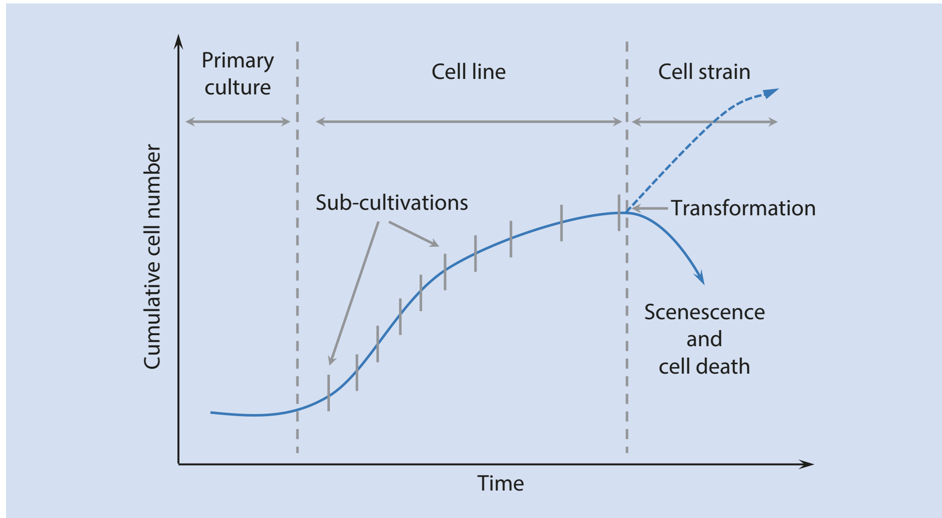


Fig. 2.10 Cell growth in vitro: primary culture, cell line, and cell strain

factors other than telomere shortening: cellular stress, long confluence, DNA damage, the presence of strong mitogenic signals (delivered by oncogenes), and other stimuli can also induce cellular senescence.

Cells cultivated in vitro can be divided into three major types: *primary cells*, cell lines, and immortalized cell strains. Primary cells are cells which are directly isolated from animal/human tissues as explant culture, with the help of enzymes or by mechanical means. The subculture of a primary cell population results in the formation of a *cell line*, which is a subpopulation of fast-dividing cells which outgrow other cells and display homogeneity in their genotype and phenotype. Such cell lines have a limited life span. But if these cells undergo a (spontaneous or manipulated) genetic transformation, they become immortal, with indefinite in vitro growth. Cell transformation is usually performed chemically or with the help of viruses. Additionally manipulated or specially selected cells from a cell line subpopulation form a *cell strain***.** A cell strain has specific properties (growth rate, tumorigenicity, etc.) and displays an even more homogeneous population than a cell line. As can be seen from Fig. 2.10, primary cells divide very slowly. In research, primary cells count as more physiologically relevant in comparison to cell lines: they are more sensitive to external signals, their genome is not altered, and they preserve many characteristics of the original tissue. The major disadvantages of primary cell culture are heterogeneity and low proliferation capacity. Cell lines provide homogeneous and pure cultures with high growth rates, which are also easy to handle (lower growth factors requirement) and cost-effective. On the other hand, cell lines show a different response when exposed to substances such as drugs, especially in comparison with primary cells or cells in vivo. This makes it difficult to translate results of drug screening experiments obtained using cell lines to in vivo studies.

During experimental planning, it is essential to evaluate beforehand all advantages and disadvantages of primary or immortalized cells in order to decide on the best cell type for the experiment. Moreover, some applications require only immortalized cell strains (e.g., biopharmaceuticals production) while in other primary cells are the only suitable choice (e.g., tissue engineering).

2.4.2 Cell Growth in Bioreactors: Important Parameters

Bioreactors provide an opportunity to scale-up cell cultivation. However, scaling-up of the cell culture creates additional challenges in terms of mass transfer. In order to achieve the optimal cultivation conditions, a suitable cultivation system/bioreactor type must be chosen, and key cultivation parameters must be optimized for each cell type and process (Fig. 2.11). First, the optimal cultivation parameters must be defined: *pH range*, *temperature*, *osmolarity*, *agitation rate*, *sparging*, *redox potential*, *metabolites*, *CO_2, and oxygen concentration*. To support homeostasis in a growing cell culture, these parameters must remain constant during the entire cultivation process. It is critical to monitor and control key cultivation parameters precisely in order to obtain high cell growth and protein production rates. Usually, suitable cultivation conditions are deduced by experiments performed in small-scale systems, where high throughput can be achieved. At the moment, the most commonly used bioreactors for mammalian cells cultivation and production of proteins, antibodies, and vaccines are the airlift and stirred tank bioreactors.

Chemical parameters pH, osmolarity, dissolved oxygen (DO), and dissolved CO_2 are considered as chemical control parameters. The physiological pH range lies between 6.8 and 8.0. The pH in bioreactor systems is usually controlled by the addition of CO_2 (sparging or overlay) and base solution. In the case of insufficient stirring, pH heterogeneity can arise. Nonoptimal stirring is also a reason for the establishment of temperature, oxygen,

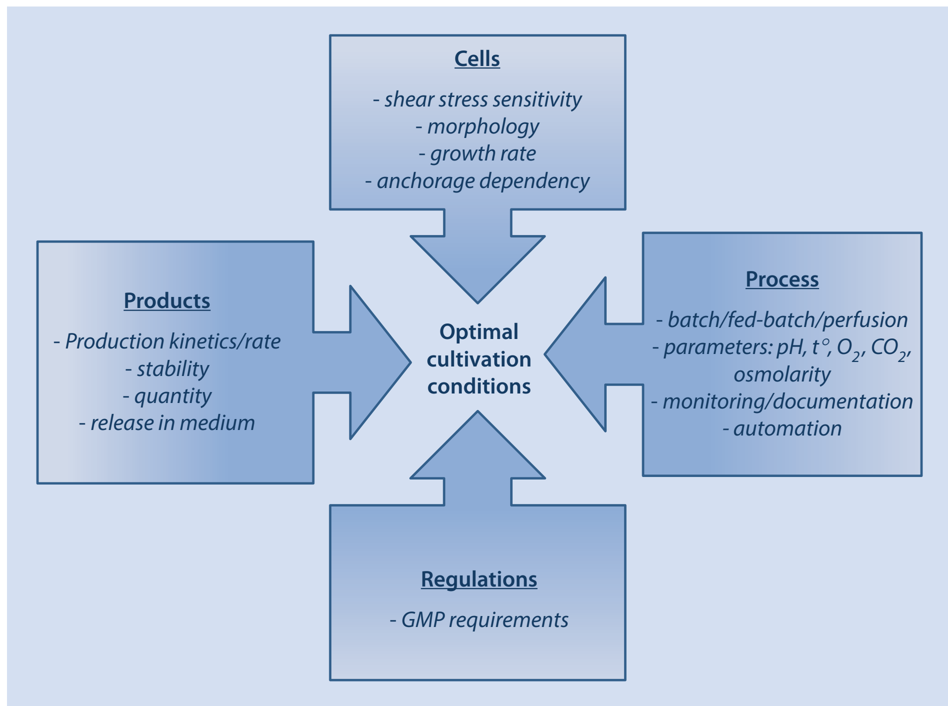


Fig. 2.11 Combination of various criteria plays an important role for the choice of the optimal cultivation system (bioreactor) and cultivation conditions

and CO_2 gradients in bioreactors. Too high concentrations of dissolved CO_2 can lead to slower cell growth and can influence product properties. Oxygen plays a key role in cell metabolism and is often required for optimal enzyme function. This means that insufficient oxygen supply will not only lead to slower cell growth and apoptosis but also will influence the maturation of the product. On the other hand, too high oxygen concentrations can also damage cells and products by an elevation of the reactive oxygen species level. The oxygen concentration balance in the bioreactor is dependent on the consumption rate and supply, both of which increase with growing cell population during cultivation. An increase of medium osmolarity during cultivation (as a result, e.g., of base addition for pH control) leads to reduced cell growth.

Physical parameters Temperature, stirring speed (agitation), and gas flow rate belong to the physical parameters. As previously discussed, efficient agitation provides better distribution of oxygen, CO_2, and temperature in the bioreactor, so the agitation speed must be as high as possible without affecting cell growth and physiology. The shear forces occurring in a bioreactor during active agitation/stirring are dangerous for mammalian cells, since these cells (in comparison to bacteria and plant cells) do not possess cell walls and are highly shear-stress-sensitive. Sparging (a method of gas supply from a sparger, a tube/device placed in the medium near to the bioreactor bottom and producing gas bubbles) represents an additional risk to mammalian cells for the abovementioned reason. While sparging provides the essential oxygen supply, it creates a situation where cells can easily be exposed to the gas-liquid interface, attach to the bubbles, and be damaged irreversibly during bubble bursting. Moreover, when the bubbles reach the top of the bioreactor, foam can be created, which is also dangerous for floating cells. In order to minimize foam formation, antifoaming reagents can be added to the medium (e.g., Pluronic F68, poly(ethylene glycol), or BSA). Furthermore, the sensitivity of cells to bubble-caused damage differs greatly depending on the cell line [14]. In general, small bubbles (less than 2 mm diameter) are more damaging than large bubbles (more than 10 mm), and large bubbles do not remain on the surface as foam [8]. The combination of sparging and impeller agitation can lead to the disruption of large bubbles from the sparger, resulting in increased cell damage.

2.4.3 Evaluation of Cell Cultures

It is important to monitor the in vitro growth and state of cells in mammalian cell culture, in order to obtain reproducible and reliable results, as well as to reveal possible contaminations and cross-contaminations on time. The routine cell monitoring in the cell culture lab is performed with the help of microscopy and flow cytometry. Additionally, various assays can be used to evaluate cell viability.

2.4.4 Microscopy

Microscopy is the oldest method for cell culture monitoring, which appeared long before the first in vitro mammalian cell cultures were established. In every cell culture lab, you can find a typical *inverted microscope* usually equipped with the phase-contrast technique. The main difference between the inverted microscope and conventional microscopes is that the objectives are located not above but under the stage. This construction makes it

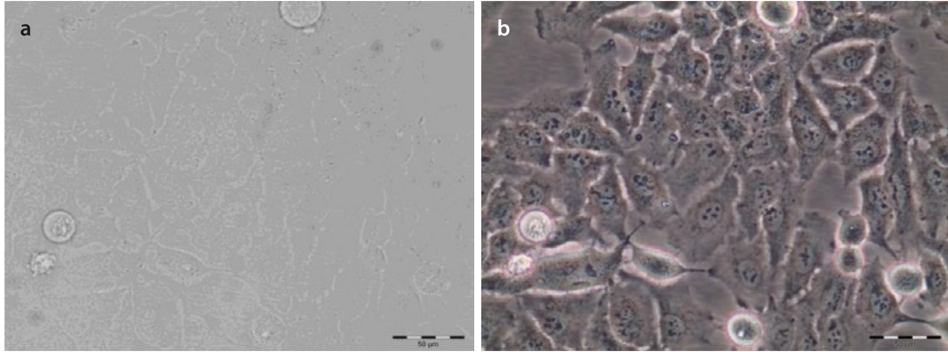


Fig. 2.12 Images of the same culture of HeLa cells made with bright field **a** and phase-contrast **b** technique

easier to observe cells growing on the flask or well bottom. In order to visualize transparent and unstained living cells, the *phase-contrast technique* is used in most microscopes. Phase-contrast microscopy is a contrast-enhancing optical technique which translates the changes in optical path length to changes of light intensity, which in turn can be detected by the human eye or with a camera. Phase-contrast technique was introduced in 1934 by the Dutch physicist Frits Zernike who received the Nobel Prize in Physics in 1953 for this discovery. In contrast to bright light microscopy, cell borders and organelles, including nuclei and nucleoli, can be perfectly seen in the phase-contrast field (Fig. 2.12).

Using microscopy, cells can be monitored in routine inspections before manipulation and the biological dynamics of the culture can be observed over time. Incubator microscopes can be used for the online monitoring of cell migration and growth in cultures. These microscopes are placed directly into incubators and can be operated at high temperatures and humidity. Using such microscopes, time-lapse videos of in vitro cell behavior can be made. Moreover, these microscopes provide the unique opportunity to monitor the cells under special cultivation conditions (e.g., hypoxia) without the necessity of taking cultures out of the incubator for the time needed to make a photo. Incubator microscopes can also be equipped with phase-contrast, bright field, as well as with various fluorescence features. On Fig. 2.13 an example of such a microscope, the Lumascope 600 (Etaluma, USA), is presented.

As an alternative to incubator microscopes, special incubation chambers can be used, which are placed on the stage of the normal lab microscope and provide cells with humidity and CO_2. The third possibility for online cell growth monitoring is the use of small incubators with integrated imaging systems. Such incubators (or live cell imaging systems) are fully automatic and can perform a simultaneous high-throughput monitoring of cells cultivated in multiple wells.

With the use of microscopy, a researcher can easily define the time point when cells must be subcultivated. After a lag phase in culture, the cells enter the exponential growth phase. Before the cells become confluent and various factors slow down cell proliferation (e.g., contact inhibition), cells are detached from the cultivation vessel, diluted (depending on the growth rate), and reseeded in a new vessel. Estimation of the growth phase using microscope is usually performed by an evaluation of the cell confluence. Typically subcultivation must be performed when confluency reaches 60–80%, which means that the cell monolayer covers 60–80% of the growth surface.

Fig. 2.13 Incubator microscope Lumascope 720 (Etaluma Inc, USA) **a**, and time-lapse videos **b** made with Lumascope, **c** inverted routine microscope (Nikon Corporation, Japan)

2.4.5 Estimation of Cell Number and Viability

After detachment with proteases, detachment reagents (e.g., EDTA) or by using a cell scraper, cells are counted either with *automatic cell counters* or with the help of a *hemocytometer*. In both counting techniques, *trypan blue* is used to distinguish between live and dead cells. After addition of the trypan blue reagent to a freshly prepared cell suspension, the viable cells remain unstained, while dead cells with a disintegrated cell membrane turn blue. The trypan blue dye can easily penetrate compromised cell membranes and stain intracellular proteins, resulting in blue-colored cells. There are two types of automatic cell counters: counters which evaluate cell number and viability in flow-through suspension samples (e.g., CASY technology, Cedex Analyzer) and counters which make a picture of

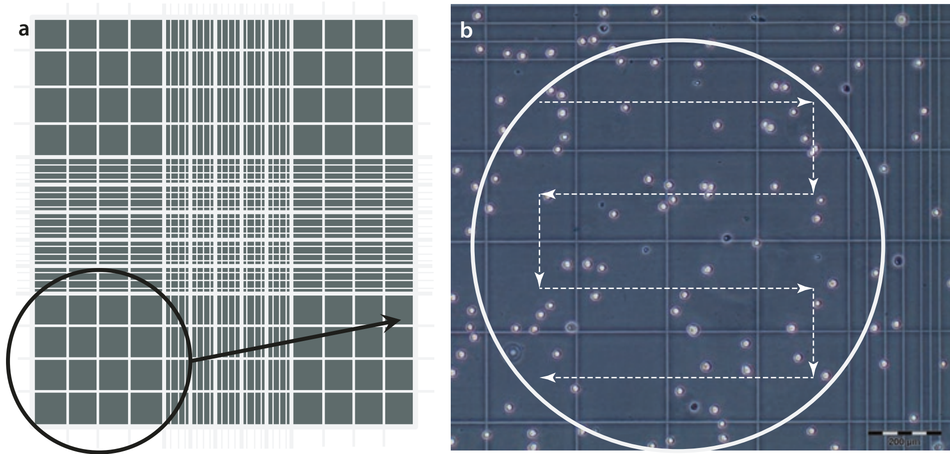


Fig. 2.14 **a** Schematic presentation of the grid structure in a hemocytometer: there are four large squares each consisting of 16 small squares; **b** Microphotograph of the cells in a hemocytometer – viable calls are unstrained and dead cells are colored blue, dashed arrows indicate the track for cell counting

the sample placed on a special slide followed by a calculation of the initial concentration (e.g., Countess® cell counter, TC20™ cell counter). Nearly the same principle lies behind cell counting with a hemocytometer: here a sample of cells is mixed with trypan blue solution and is loaded in the space between a hemocytometer with a special grid (Fig. 2.14) and a cover glass. Cells in the squares are counted, and the initial cell concentration in suspension is calculated (e.g., for the chamber with depth of 0.01 mm, the estimated cell number in one large square is multiplied by 10,000 to obtain the cell number per ml).

Another approach to cell viability estimation is live/dead assays: here one dye is used to stain viable cells (e.g., Calcein-AM) and another to stain dead cells (e.g., propidium iodide). The advantage of this method is its quickness (10–15 min), and the main disadvantage is that it is semiquantitative. Indirect cell viability assays are based on measurements of cell metabolism. In this type of assays, a solution with a substrate is incubated with cells for a certain period of time, during which it is, e.g., reduced by intracellular enzymes, and the concentration of the product is measured afterward. Substrates and products can be measured by a colorimetric (MTT assay) or fluorescent assay (Alamar blue assay). *Flow cytometry* is widely used in cell culture for different applications including immunophenotyping (specific cell surface marker expression), apoptosis analysis (annexin V, DNA fragmentation, mitochondrial membrane potential), cell cycle analysis (BrdU incorporation and PI, Hoechst 33,342 or DAPI staining of DNA), proliferation analysis (cytoplasmic dyes which are diluted with every cell division), transfection efficiency, gene expression, and particle uptake.

Take-Home Messages

- In vitro cell cultivation started already more than 100 years ago.
- One of the major requirements is the necessity to work under aseptic conditions.
- There are several types of biological contaminations in the cell culture: the most difficult ones are invisible contaminations with viruses and mycoplasmas or cross-contaminations with other cells.

- In conventional cell culture vessels, there are several types of plastic surfaces for cell adhesion.
- Bioreactors enable cultivation under dynamic conditions as well as possibility for upscale.
- The most important parameters of cell growth are doubling time and growth phase.
- Optimal chemical and physical parameters during cultivation in bioreactor enable best possible cell growth and protein production.

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Cell Culture Media

Reinhard Henschler

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This chapter is dedicated to my former Ph.D. student, Dietrich (Dieter) Möbest who brought with him much basic knowledge and a high interest in cell culture media. Due to a severe illness, Dieter could not further pursue his career after his postdoc time. His solid knowledge in biotechnology and specifically cell culture media and his constant optimism stimulated my own interest in the field and formed the basis of several joint studies investigating the role of culture medium in determining the fate of hematopoietic cells.

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What You Will Learn in This Chapter

You will learn the basic constituents of culture media for mammalian cells and their derivation. The historic development of constituents and additives to culture media such as amino acids, protein, vitamins for individual cell types from different tissues will be covered, with a focus on stem and progenitor cells. You will read on approaches towards the derivation of defined media, and the supplementation of serum additives. The relevance of physical parameters such as osmolarity and buffer systems will be approached. You shall gain an understanding on why essential components of cell culture media, of which several are in general use worldwide, have been added and assess the relevance of their presence in given cell culture settings.

3.1 Purpose of Cell Culture Media

The ability to preserve and amplify different cell types from animal and human tissues has been a key prerequisite for a multitude of discoveries in modern cell and molecular biology, including the identification of chromosomal aberrations in cancer and the production of monoclonal antibodies.

Cell culture media are the major constituent providing an environment securing the survival, further continuous propagation, and/or differentiation for cells that have been either freshly explanted from an organism or have been transformed from other primary cultures. Cell culture media shall in the first line provide the energy sources, confer oxygen to cells, contain a salt composition and pH which are beneficial to the cultured cells, and take up metabolites and debris of cultured cells. In addition, culture media are often used to provide signals for the survival, growth, and/or differentiation of the cultured cells. Thus, cell culture media have to replace the natural environment of the cultured cells in fluid form.

Experiments to isolate cells in culture date back in the early twentieth century, trying to establish expanded cells in plasma clots or fibrinogen clots and allowed for the preservation and isolation of different cell types such as neural tissue or bone marrow [23, 24]. First efforts toward the use of chemically defined media and continuing multiplication on serial subculture included studies by Fisher et al. [15] who supplemented a defined medium with dialyzed chicken plasma, serum, and embryo extract [15]. A multitude of different ingredients derived from tissue, blood, or organ extracts had been tried and established to isolate mammalian cells, including human cells. Among these were plasma clot culture, fibrinogen clots, embryonic cell extracts, and supernatants from explanted cells or tissues. ◘ Table 3.1 lists important purposes of cell culture media and typical basal ingredients mediating these functions.

3.2 Minimum Requirements for Culture Media for Mammalian and Human Cells

In 1955, Harry Eagle published pivotal work on the way to defined cell culture medium. He described a minimum of 27 different factors which needed to be present in "essential" medium (termed therefore, Eagle's Minimum Essential Medium) [13]. The selection of these ingredients, which included inorganic salts, glucose, amino acids, and vitamins, was justified by data omitting the respective reagent. The definition of these minimal ingredients laid the basis for the continuous and stable propagation of murine (L fibroblasts) and

Table 3.1 Main purposes of cell culture media and ingredients

Purpose	Typical ingredient
Energy source	Glucose
Confer oxygen	(free diffusion)
Provide ionic strength	NaCl, KCl, CaCl, $MgCl_2$
Maintain physiological pH	$NaH_2PO_4 * H_2O$, $NaHCO_3$
Take up metabolites and debris of cultured cells	(free diffusion/suspension) added serum or albumin (in part)
Provide survival/growth differentiation signals	(dialyzed) human, horse, or calf serum

Table 3.2 Essential constituents of basal media for cultivation of the HeLa cell and mouse fibroblast

Inorganic salts	Amino acids	Vitamins
NaCl (100 mM)	Arginine (0.1 mM)	Biotin (10-3 M)
KCl (5 mM)	Cysteine (0.05 mM)	Choline (10-3 M)
$NaH_2PO_4 * H_2O$ (1 mM)	Glutamine (2.0 mM)	Folic acid (10-3 M)
$NaHCO_3$ (20 mM)	Histidine (0.05 mM)	Nicotinamide (10-3 M)
$CaCl_2$ (1 mM)	Isoleucine (0.2 mM)	Pantothenic acid (10-3 M)
$MgCl_2$ (0.5 mM)	Tyrosine (0.2 mM)	Thiamin (10-3 M)
Carbohydrate	Leucine (0.2 mM)	Pyridoxal (10-3 M)
Glucose (5 mM)	Methionine (0.05 mM)	Riboflavin (10-4 M)
Other	Phenylalanine (0.1 mM)	
Serum (horse, fetal calf; 1–10%)	Threonine (0.2 mM)	
	Tryptophan (0.02 mM)	
Penicillin, streptomycin	Tyrosine (0.1 mM)	
Phenol red	Valine (0.2 mM)	

Adapted from Eagle [13]

human (HeLa uterine carcinoma) cell lines previously described by Sanford et al. [24] and Scherer et al. [41], allowing their growth also on glass culture surfaces. The essential constituents consisted of salts (NaCl, KCl, $CaCl_2$, $MgCl_2$, $NaHCO_3$, and NaH_2PO_4), glucose, 13 essential amino acids, and a number of vitamins of the B complex. Table 3.2 lists the ingredients of Minimum Essential Medium according to Eagle [13].

Careful experimentation by omitting single constituents demonstrated that each constituent is essential. A number of different carbohydrates could however substitute for

glucose: Galactose, mannose, and maltose were almost as active as glucose; some other sugars turned out to be only slightly less active. In the vitamin section, flavin adenine dinucleotide (FAD) could substitute for riboflavin, diphosphopyridine nucleotide (DPN) or triphosphopyridine nucleotide (TPN) for nicotinamide, coenzyme A for pantothenic acid, and cocarboxylase for thiamine. Phenol red was included to visualize changes in pH into the acid or alkaline direction which would lead to nonphysiological conditions. Especially, alkaline reactions would be encountered if CO_2 concentrations in the incubators fell below efficient levels.

As to serum, either horse or fetal calf serum was added, which usually had to be pretested on a batch basis. Serum concentrations between 1% and 5% were recommended for survival/experimentation, whereas 5–10% were considered to be useful for propagation of cultures. Alcohol-salt precipitation experiments proved that part of the serum constituents were inert, while others were only weakly active. When serum was exhaustively dialyzed, it was also inactive. However, serum fractions obtained by simple salting out with $(NH_4)_2SO_4$ followed by a 24-h dialysis were all active, to similar degrees.

Further experiments by Eagle demonstrated that cellular functions other than proliferation were less sensitive to the composition of the medium and the presence of the described “essential” components [14]. For example, the amount of poliomyelitis virus released into the medium by the HeLa cell line was quantitatively not affected by the omission of either serum protein, amino acids, or vitamins from the medium shown in ◘ Table 3.2. However, omission of either glucose or glutamine from the medium resulted in a marked decrease in virus production.

3.3 Natural Sources of Culture Media Ingredients

The results of the groundbreaking experiments in the 1950s by Eagle which defined 27 essential media components are still valid today in mammalian and also human cell culture. However, important factors could at that time not be substituted and remained unknown or poorly defined. For some constituents, this is still the case today.

Before the definition of essential medium components, such sources had been human- or animal-derived plasma, serum, lymph, extracts from adult tissues or embryos, or amniotic fluid. A main limitation of the natural media sources was and continues to be the variability between different collections (batches) and different organisms. Other limitations of serum as additive in culture medium are that they are non-defined and can contain, in addition to soluble ingredients, microparticles or exosomes which are difficult to remove. Animal-derived serum is also principally not suitable to culture cells or tissues which are used for transplantation in humans for more than one application, due to the formation of immune responses against animal proteins in patients after transplantation of cells which had been expanded in the presence of, e.g., calf serum [19].

Thus, in serum-supplemented culture, important stimuli continued to be provided by addition of little defined, natural sources. The variety of stimuli provided by serum is summarized in ◘ Table 3.3. Further functions of natural ingredients in cell culture media include activities to “detoxify” the culture, due to binding and inactivation of waste products, to provide (colloid) osmotic pressure for cultured cells, to increase the viscosity of medium and protect cells from mechanical damage, and to buffer against unphysiological pH.

Table 3.3 Substances and activities contained in serum preparations used as additives in cell culture media

Amino acids	Vitamins	Carbohydrates	Trace elements
Lipids	Hormones	Growth factors	Minerals (e.g., Na+, K+, Zn2+, Fe2+)
Binding and transport proteins (e.g., albumin, transferrin)	Attachment and spreading factors (e.g., fibronectin)	Protease inhibitors (protect cells from proteolysis)	Proteases

3.4 Amino Acids

As described above, Eagle [13, 14] worked out and published a list of essential amino acids which allow for the continuous growth of several established cell lines in 2D culture systems. These essential amino acids are also used for the synthesis of nonessential amino acids and other metabolic intermediates such as phospho-l-tyrosine, S-sulfo-l-cysteine, or branched amino acids [17, 40]. L-serine and glycine are involved in the metabolism of nucleic acid precursors through the tetrahydrofolate cycle. Depletion of L-serine may be overcome by additional supplementation with glycine, which leads to L-serine production, avoiding slowing of the tetrahydrofolate cycle and one-carbon metabolism, and inhibition of cell proliferation [11, 22].

Amino acids have been found to be of limited stability in solution. Therefore, for every culture medium preparation, half-life studies have to be performed to determine the intactness of amino acids when storing medium at 4 °C. Alternatively, powdered media formulations have been developed which allow longer pre-use storage. In particular, L-glutamine is instable in aqueous solutions and decomposes to form cyclic pyrolidonecarboxylic acid with the release of ammonia [8, 35]. L-glutamine can be replaced with the dipeptide L-alanyl-L-glutamine (Glutamax), and L-cysteine replaced by *N*-acetyl-L-cysteine or *S*-sulfo-L- cysteine [32, 44]. Addition of more L-glutamine than necessary results in the buildup of ammonia which can be deleterious to some cell lines. Use of phospho-L-tyrosine in place of L-tyrosine has increased solubility of this more lipophilic amino acid [40, 47].

In addition, amino acids are capable of forming mixed crystals, especially amino acids with similar side chains, and cations can form coordination modes through interactions with the nitrogen in the amino group, the hydroxyl oxygen in the carboxyl group, and the carbonyl oxygen in the carboxyl group [21]. Although the composition of amino acids are generally fixed in the established culture media, for fed batch and perfusion cultures, determination of optimal concentrations of amino acids is often pursued, and depends on the metabolic requirements of the cells used [39, 45].

3.5 Proteins, Peptides, and Lipids: The Key to Development of Serum-Free Media

When using classical cell culture media which are described below in more detail, usually sufficient amounts of plasma proteins are supplemented through the addition of serum. In comparison with adult calf serum and horse serum, fetal serum is a relatively richer

source of growth factors and is appropriate for cell cloning and for the growth of fastidious cells [2]. Adult calf serum, on the other hand, is used in contact-inhibition studies because of its lower growth-promoting properties. For primary cell cultures, such as the culture of epidermis equivalents for skin explants, the substitution of single individual factors may suffice [26].

In cases where xenogeneic components are unwanted such as the ex vivo expansion of human cells for therapies, human additives, e.g., human serum, may be used since allosensitization against animal proteins needs to be avoided. One example is the ex vivo expansion of human hematopoietic stem and progenitor cells. Another example, human mesenchymal stromal cells (MSCs) which are used as cellular therapies in a variety of clinical diseases (for review, see [7]), human platelet lysate (HPL) has been established as an efficient additive to replace serum. HPL is at the same time a rich source of human growth factors which are released from platelets during the preparation process and proved to be an efficient replacement for animal or human serum in culture expansion for MSCs [3, 6].

The ultimate aim for the in vitro growth of human hematopoietic stem and progenitor cells has been a defined medium, without addition of serum or human plasma. Thus, the challenge has been the complete omission of serum. The development of serum-free media took a decisive turn when it was possible to provide three main constituents of serum in recombinant or purified form:

1. Albumin
2. Transferrin
3. Cholesterol (or other types of a lipid source)

The most commonly supplemented proteins and peptides in cell culture media are albumin, transferrin, and fibronectin. Albumin is the main protein in blood acting to bind water, salts, free fatty acids, hormones, and vitamins and transport them between tissues and cells. Albumin is also a key component securing the binding of added growth factors such as recombinant cytokines into serum-free media. Both transferrin (expressed in rice) and cholesterol are available in synthetic form as media supplements.

Today, a large number of serum-free cell culture media are on the market and in routine use, including culture media designed for propagation and/or differentiation of hematopoietic cells. By substituting of key functions of serum by the addition of albumin, transferrin, and a lipid source instead of serum [38], serum-free cultures could be established for cell lines and a number of primary hematopoietic and other cell types Arora et al. [2]. For example, serum-free medium for ex vivo expansion of hematopoieitic cells can be manufactured using as a basis an established basal medium such as Iscove's Modified Dulbecco's Medium (IMDM; explained below) [30]. Generally, recipes for such media are proprietal. A rather comprehensive list of FBS-free media for use in mammalian and human cell culture is given by the FRAME Initiative [16].

3.6 Vitamins

As shown above, the water-soluble B-group vitamins are present in the essential culture medium and are needed for cell growth stimulation. In contrast, lipid-soluble vitamins such as Vitamins A, D, E, and K are classically added to media through serum. The seven vitamins which have to date proved essential for the survival and multiplication of mouse

L cells and human HeLa carcinoma cells are choline, folic acid, nicotinamide, pantothenic acid, pyridoxal, riboflavin, and thiamin. Omission experiments showed that if cells lacked these vitamins for 5–15 days, the deficiency became apparent by the cessation of cell replication and the development of specific cytopathogenic effects. These changes could be reversed by adding the missing vitamin. The maximally effective concentrations were elaborated for L cells and HeLa cells (Eagle et al. 1955a, b).

The stability of vitamins in culture media is however variable. Kurano et al. [25] found a relative instability of ascorbic acid and thiamine from alpha-modified Eagle's Minimum Essential Medium (MEM-alpha) when it was supplemented with 10% fetal calf serum. Vitamin B_{12} is present in sera at variable concentrations depending on species and due to its chemical instability is vulnerable to storage and handling. A wide range of Vitamin B_{12} is present in formulations of classic media. Basal media often contain no vitamin B12, RPMI-1640 and Iscove's Modified Dulbecco's Medium (IMDM) contain low levels (3–10 nM), and alpha-MEM contains 100 nM vitamin B_{12}, whereas H-Y Medium and McCoy's 5A Modified Medium contain 923 nM and 1.48 µM of vitamin B_{12}, respectively [40]. In general, hematopoietic stem and progenitor cell cultures contain primary, freshly isolated cells and have a limited ability to self-renew or even transform [12, 27]. The limited life span can explain a relatively low need for vitamin supplementation during their culture expansion. Vitamins, particularly lipid-like Vitamins A, D, E, and K, are transported by and bound to albumin. Importantly, the source and the purification grade of the albumin preparation added, especially to serum-free cell culture medium, determine the content of vitamins.

3.7 Buffering Systems

The gaseous CO_2 balances with the CO_3/HCO_3 content of culture medium. Cultures with a natural buffering system need to be maintained in an air atmosphere with supplementation of 5–10% CO_2 is usually maintained by an CO_2 incubator. This natural buffering system is practical, economical, and nontoxic [20, 37] and remains the major method to control pH in cell culture systems today.

4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) is a chemical buffer using a zwitterion. It shows a superior buffering capacity in the pH range 7.2–7.4. A controlled gaseous atmosphere is no longer required [42]. In contrast to the bicarbonate system, HEPES is relatively expensive. For some cell types, it may be at higher concentrations. HEPES has been found to enhance the sensitivity of media to phototoxic effects induced by exposure to fluorescent light [46].

pH Indicators Most of the commercially available culture media include phenol red as a pH indicator, which allows constant monitoring of pH [36]. When cells grow and accumulate acid metabolites, a color change reflecting lowering of the culture pH is taking place toward orange and later yellow. At higher pH levels, the color turns purple. Media are bright red at pH 7.4, the optimum pH value for most cell cultures. Disadvantages of using phenol red include the ability of phenol red to stimulate steroid hormone receptors, particularly estrogen [4]. Thus, media without phenol red should not be used with estrogen-sensitive cells, e.g., from mammary tissue. Phenol red can also, when used in some serum-free formulations, interfere with the sodium-potassium homeostasis [18].

3.8 Basal Medium Formulations

In the following, the main characteristics of some basic cell culture media are described. Many of them are available as powder or in liquid form. Powdered media often do not contain sodium bicarbonate, because it tends to gas off and require the addition of three sodium bicarbonate upon dissolving it in water (3.7 g/L).

3.8.1 Eagle's Minimum Essential Medium (EMEM)

EMEM is one of the first widely used standardized media, formulated on his description of media, and was formulated from his basal medium described above [13, 14]. EMEM contains bicarbonate buffer, salts corresponding to the extracellular milieu (i.e., blood plasma), a number of essential amino acids, and sodium pyruvate. EMEM is usually supplemented with additional components and serum. It has been found suitable for a wide range of mammalian cells.

3.8.2 Dulbecco's Modified Eagle's Medium (DMEM)

Compared to EMEM, DMEM is richer, containing almost twice the concentration of amino acids and four times the amount of vitamins. Moreover, ferric nitrate, sodium pyruvate, and additional amino acids are included. Originally, it contained glucose at 1000 mg/L, but a variation with 4500 mg/L glucose has been proved to be better to culture a number of cells. As is EMEM, DMEM is a basal medium and does not contain proteins or growth-promoting agents and requires addition of fetal calf serum (FBS). DMEM contains sodium bicarbonate buffer system (3.7 g/L) and required the presence of CO_2 to maintain the required pH. DMEM is applied widely for culturing primary mouse, chicken, and human cells including fibroblasts. It is used as a basal medium also for embryonic stem cells.

3.8.3 Roswell Park Memorial Institute (RPMI)-1640

RPMI-1640 is a medium applied for many mammalian cells; it has been developed especially for hematopoietic cells and peripheral blood lymphocytes [31]. RPMI-1640 also uses bicarbonate buffering system, but differs from the most mammalian cell culture media by its ability to maintain a pH of 8.

3.8.4 Iscove's Modified Dulbecco's Medium (IMDM)

IMDM is a modification of Dulbecco's Modified Eagle Medium. Includes selenium as well as additional amino acids and vitamins. It lacks iron, with potassium nitrate replacing ferric nitrate. IMDM contains no proteins, lipids, or growth factors and required addition of serum. However, serum-free formulations have been derived from IMDM [30].

3.9 Co-culture with Nurturing Cells and Conditioned Media

Especially in the case of hematopoietic cells, cocultivation with other cells has been a major step leading to the successful growth of lymphocytes and immature hematopoietic cells in culture. Miller et al. [28] published the use of a leukemic cell line in the establishment of lymphocytes. Later, it was found that Epstein-Barr virus (EBV) was the transforming agent for the primary cells [29]. Also in the 1970s, the group of T.M. Dexter in Manchester together with colleagues found that bone marrow, when it was successfully established in culture, i.e., produced mature progeny from immature hematopoietic precursor cells, always contained a stromal cell layer containing adventitial reticular fibroblasts, endothelial cells, and macrophages which formed contact-dependent support of proliferating stem and progenitor cells [10]. Interestingly, hyperosmolaric conditions were beneficial in these culture systems. Moreover, spleen cells from other species could take over the support function for human hematopoietic stem/progenitor cells [1].

In cultures of hematopoietic cells, more recently it has been shown that removal of accumulating growth-inhibiting soluble cytokines can result in improved culture conditions. One relevant cytokine which inhibits hematopoiesis is transforming growth factor (TGF)-β1. By maintaining the concentrations of TGF-β1 below an upper threshold throughout hematopoietic cell culture, ex vivo expansion of hematopoietic progenitor cells could be enhanced and established over longer culture periods [9]. For other factors secreted into the medium, differential effects were shown. Bone morphogenetic protein 4 (BMP-4) at 10 ng/mL, but not at lower concentrations, was permissive for the emergence of cells capable to form not only hematopoiesis but also endothelial cells [34]. Early addition of vascular endothelial growth factor (VEGF)-2 positively influenced this development, whereas later addition did not. This example illustrates that addition and removal of growth factors during culture from the medium bears potential to design the outcome.

Finally, stem cells themselves may be a source of growth and differentiation inducing activity which can be used in culture media. Thus, conditioning of media for pluripotent stem cells by other stem cell types has recently entered the field of stem cell research and may widen the options of stem cell culture, as well as its use in diagnostics, research, and the development of therapies from cultured cells [5, 33, 43].

Take-Home Messages

- Cell culture medium requires essential salts, amino acids, vitamins, and energy sources. Most standardized media require addition of animal or human serum.
- Metabolites that are not further processed or toxic need to be taken up.
- Medium may be designed to provide specific biological signals to cultured cells, relevant for their survival, proliferation, and differentiation.
- Replacement of serum needs imposes a major challenge to medium development and may be accomplished through addition of albumin, transferrin, and a lipid source.

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Establishment of Tumor Cell Lines: From Primary Tumor Cells to a Tumor Cell Line

Katharina Meditz and Beate Rinner

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What You Will Learn in This Chapter

The present chapter will give an overview about handling of tumor tissue, isolation of tumor cells, and finally the establishment of tumor cell lines. Characterization and identification of established cell lines will be shown, on the basis of three examples: clival and sacral chordomas and NRAS-mutated melanoma. Understanding the culture conditions will help to find the perfect requirements for the culturing of each different cell line. Contamination in the cell culture is a touchy topic; detection and elimination of microbial and cell-cell contamination will be discussed in detail. By adhering some golden rules, success of cell line establishment will be guaranteed!

4.1 Importance of Primary Tumor Cell Culture: As Close to In Vivo as Possible

George and Margaret Gey spent almost 30 years trying to establish an immortal human cell line, in order to understand the biology and mechanism of tumors. After establishing the Tissue Culture Laboratory, George Gey made his monumental breakthrough – he isolated the first tumor cell line from a cervix carcinoma which he named HeLa. George Gey always said: "The key to cancer is right at our fingertips - if we could only reach out and grab it" [12, 26]. Sadly he died from a pancreatic tumor.

Since the discovery of cell lines, their importance has continued and has even increased. The reasons are as follows: (i) they are easy to handle, (ii) they offer unlimited self-replication, (iii) they have a relatively high degree of homogeneity, and (iv) they are easily replaceable from frozen stocks. There is no positive without some negative because (i) cell lines are prone to genotypic and phenotypic drift during passaging, (ii) cell lines don't store well over many years, (iii) cell line subpopulations cause phenotypic changes, and (iv) cell lines from different labs show different karyotypes and therefore reproducibility might be lacking [4].

4.1.1 Cell Culture Definition

Primary culture is the initial culture taken directly from the tissue; it mimics the situation most closely to the original tissue and is fundamental for research. Cells can migrate and grow directly from the tissue (explant technology), or cells can be enzymatically or mechanically isolated from the tumor tissue. In relation to their growth behavior, cells can grow in vitro as adherent culture or they can grow in suspension.

After the tumor cells grow out and cover the whole cell culture flask, cells must be transferred into a new cell culture flask, the nomenclature of primary tumor cells changes, and it becomes a cell line. There are two classes of cell lines, the continuous cell line with indefinite population expansion or finite cell lines with a defined life span. Currently, there is a wide range of different cell lines in cell banks, which are well characterized. However, it is still useful to establish new cell lines, because heterogeneous tumors require different cell line systems to investigate the biology of the tumor. Cell lines that have been very long in culture can change their genetic and phenotypic characteristics.

4.2 A Long Way from Tissue to the Desired Cells

From tissue to the desired cell can take a lot of time and can also be very tricky; depending on the growth behavior of the cells and the quantity of existing tumor cells in the available tissue. This chapter provides a general overview of tumor tissue, media, and cell isolation. By using contrasting examples in terms of tumor growth, the establishment of a cell line named MUG-Mel2 from a very aggressive NRAS melanoma tumor and the establishments of very slow-growing chordoma tumors, named MUG-Chor1 and MUG-CC1, will be explained (Figs. 4.1 and 4.2).

4.2.1 Tumor Tissue

The translation of the Latin word tumor is "swelling." The distinguished pathologist Wallace H. Clark gave an excellent definition of the word tumor: "A tumor is a population of abnormal cells characterized by temporally unrestricted growth and the ability to grow

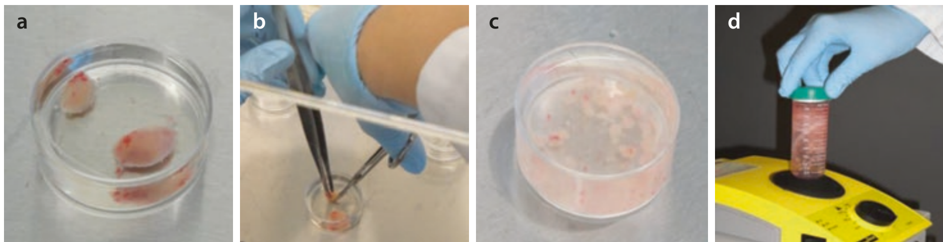

Fig. 4.1 Establishment of a tumor cell line. The tumor tissue should be obtained immediately after surgery, surrounding tumor tissue is removed, and to avoid contamination an antibiotic bath for 10 min in PBS + 10% PS should be done **a**. After the antibiotic bath, transfer the tumor tissue into PBS or media, and the tissue is cut into very small pieces (1–2 mm^3) by scissors or scalpels **b**. After disaggregation of the tumor tissue into small pieces **c**, the suspension is collected, and tumor pieces in PBS or media will be centrifuged. If provided in the protocol, vortex the minced tumor tissue **d**, and different fractions should be set in suitable media

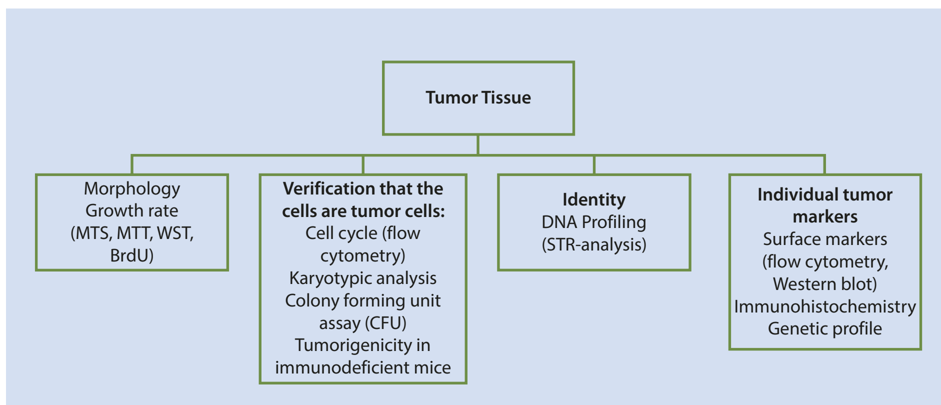

Fig. 4.2 Overview of a characterization of a new established tumor cell line

in at least three different tissue compartments - the original compartment; the mesenchyme of the primary site (tumor invasion); and a distant mesenchyme (tumor metastasis)" [5, 7]. Solid tumors are structured as parenchyma (neoplastic cells) and stroma cells (surrounding tumor cells). Tumors with epithelial origin can show a separation of these compartments with a basal lamina [7]. That means tumors do not exist only of malignant cells, but many other cells as well. Also essential is the tumor microenvironment (TME). Depending on tumor entity, the TME can consist of cells of the immune system, vascular and lymphatics cells, fibroblasts, pericytes, and adipocytes in vastly different amounts [2]. Some cells are easy to distinguish by typical morphology, like the epithelial fraction, predominantly single cells with classical cobblestone morphology, and stromal fraction consisting of fibroblast cells, cells with a bipolar spindle shape typical for fibroblasts; immune cells present spheroid cells above the adherent cells or mast cells/macrophages.

4.2.2 Four Steps to Success

In the first step, the condition of the tumor tissue is of great importance. Short ischemia times are an advantage, but you can isolate cells from a tissue 24 h after surgery, as long as the tissue is kept cold (4 °C) in media. The transport of tissue from surgery to the lab should be in media without any additives, such as sera (FBS) or antibiotics, to prevent it from drying out. Being FBS-free is important to inhibit cell growth during transport. Antibiotic-free, because in the lab each tissue will be incubated for 10 min in a 10 time antibiotic bath to avoid contamination and if you have antibiotics in the transport media you cannot be sure of the antibiotic concentration and incubation time. Living cells can only be isolated from fresh viable tumor tissue.

The second step in creating cell lines is to know what kind of cells you want to isolate and then to keep track of them! A good instinct is necessary for the successful establishment of cell lines.

In the third step, each tumor requires its own isolation method which, as well as medium, can be found in most literature. A very widespread method is the mechanical dissociation or cutting the tissue with scissors or sharp blades into very small pieces – usually 1–2 mm^3, with the option of homogenization by filtration through a nylon filter (50–100 μm opening), vortexing and repeating those steps to get rid of dead cells, debris, and various different cell types.

One must be aware that during isolation of tumor cells, there can be a varying amount of non-neoplastic cells that will complicate tumor cell line establishment. Therefore it is important that a histological section of the tumor is examined by a pathologist to determine the proportion of tumor cells present, as this will affect the success of cell line growth. Enzymatic dissociation is also a way to separate single cells: there are different enzymes that are suitable for dissociating cells from solid tumor and to digest minced tissue into single cells. The concentration, temperature, and incubation time of the enzyme to preserve the cell viability and integrity are important. There are various enzymes available including collagenase, trypsin, papain, and elastase. Each enzyme with distinct target specifies with an affinity for different tumors [19].

In the fourth step, each cell needs its own media; therefore a thorough research of all literature is a prerequisite before starting any cell line establishment. There is a wide range of available basic media, which can be adapted by different additives to the needs of the cells. However, in the production of specific media, care must be taken that the osmolality

is maintained. An increase of FBS, for example, from 10% to 30%, can strongly influence the osmolality of the medium. By comparison, the osmotic value of human blood is 300 mOsmolkg-^{1}, and the osmotic value in most of the basic media is 280–340 mOsmolkg^{-1} [3]. There are different ways to facilitate the adherence or growth of cells with the help of additives in the media, for example, ITS, a mixture of recombinant human insulin, human transferrin, and sodium selenite. Adherence of cells can also be reinforced with coating of cell culture flasks with various enzymes, like fibronectin or Matrigel.

4.2.3 Protocol: Tumor Cell Line Establishment for Adherent Growing Tumors

- Preparation of tumor tissue: removal of non-tumor tissue (fatty tissue, blood clots, and connective tissue) by sterile scissors or scalpels.
- To reduce contamination risk, an antibiotic bath, 10× PS solution, with incubation for approximately 10 min is recommended.
- Transfer the tumor tissue from antibiotic bath to a PBS or media.
- Mechanical and/or enzymatic disaggregation with sterile forceps and scissors or scalpels of the tumor in very small pieces to get more surface for outgrowing of the tumor cells.
- Optional: Vortexing of the tumor pieces.
- Centrifuge and remove the supernatant.
- Resuspend the pieces with appropriate media in different cell culture flasks (due to the filter of the flasks, cells might have a better outgrowth than in, e.g., 6-well plates)
- Incubation of the cells in a humidified incubator at 37 °C in an atmosphere of CO_2 (amount of CO_2 depends on the used media).
- Regularly morphological observation of the tumor cells.
- When the whole cell culture flask is overgrown and the media is consumed, subcultivation (splitting) is required.
- Subcultivation: detachment of the adherent monolayer by the use of proteolytic enzyme like trypsin or more gentle with Accutase. Cell-cell contact is broken and cells are in suspension; after centrifugation cells can be seeded into new flasks with appropriate cell amount and new fresh media to continue cell growth.

4.2.4 Characterization

There are a large number of different cells in the tumor tissue, so it is very important to characterize the outgrowing cells as quickly as possible. One characteristic of cancer cells is replicative immortality, which means that cancer cells can divide many times more than normal cells. Very often only fibroblasts develop and grow over the tumor cells. Fibroblasts also have the ability to remain for long in culture, up to 50 passages in culture. The problem with fibroblasts is that they are not always recognizable by their morphology and fibroblasts are often mistakenly confused with tumor cells. For those reasons the tumor potential has to be tested as soon as possible. In most cases only few tumor cells are available at the beginning, and the characterization method must be adapted to the number of cells. During the first growth phase, only morphological observation is possible. The established cell line should have the same characteristic as the origin tissue, whereas, for

example, mesenchymal origin can be detected with vimentin, epithelial characteristic with cytokeratin. To check the tumor potential of the cells, a colony-forming unit assay can be done; this assay requires only a small number of cells and indicates the tumor potential of a cell through clone growth. Tumor cells are indicated by aneuploidy and chromosome instability, cell cycle analyses by flow cytometry can be done to reveal the ploidy state of the cells, whereas karyotype analyses reveal the chromosome instability of the tumor cells. Tumor marker and genetic profiles should remain constant expressions during cultivation; however, some markers can be lost during cultivation. To confirm that the established cell line has been developed from the tissue of origin, a DNA profiling by STR analyses to prove the identity of the tumor is highly recommended.

4.3 General Tips About the Establishment of Tumor Cell Lines

In general, to generate tumor cell lines, cells should (i) be regularly cryopreserved, (ii) be passaged more than 100 times and retained in culture for more than 12 months, and (iii) mainly keep their genetic phenotype and morphology during cultivation.

4.3.1 Hayflick Problem

One problem during cultivation is the so-called Hayflick limit. Leonard Hayflick and Paul Moorhead discovered that human cells derived from embryonic tissues could only divide a finite number of times in culture [14]. Stages of cell culture were divided into three phases. Outgrowth of cells from an explant or tissue was named phase I. Division of cells represents phase II. The cells then proliferate in culture, depending on the cell type, and after a certain time period, cells start to divide more slowly and can also stop dividing, and this is defined as phase III. Hayflick and Moorhead described the phenomenon of growth arrest as the Hayflick limit or replicative senescence. If cells decrease or stop dividing, they should be transferred to a new flask with a higher cell density, FBS concentration can be increased for a short time, and it is highly recommended that only 50% of the media is changed, in order to keep autologous growth factors in culture. It's much easier to cultivate fast-growing cells than slow-growing cells; however, cells can stop dividing without any conceivable reason.

4.3.2 To Get Rid of Other Cells

Mentioned in ▸ 4.2.1 a lot of different cells are in a tumor tissue. To get rid of non-tumorigenic cells, selective adhesion and detachment techniques can be used [10, 23].

Fibroblast and tumor cells present a different density and size; after trypsinization, cells can be transferred to a new cell culture flask. After a time period (e.g., 10 min–2 h), certain cells will settle to the bottom, remaining media should be moved into a new flask, and the processes can be repeated (◘ Fig. 4.3).

It is also very useful to take different fractions of cells isolated from a tumor, as in the example, for the establishment of MUG-Chor1. Keep all fractions in culture and hope that in one fraction tumor cells will start to grow! The fibroblast separation described above together with the fractions taken can mean more than 30 flasks in the incubator at one

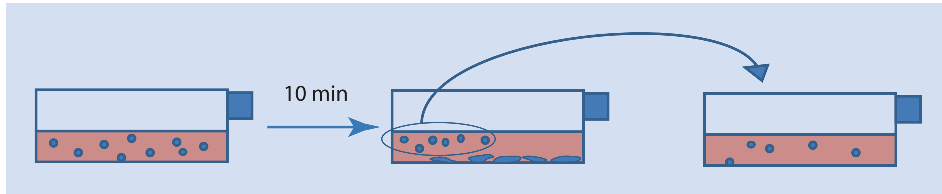

Fig. 4.3 Easy separation of different cell types due to different size and density

time during cell line establishment. Therefore a complete description of each flask needs to be recorded. The most important point of primary culture is do not discard anything during cultivation and be patient!

In the next passage, the establishment of three tumor cell lines will be discussed.

4.4 Establishment of Tumor Cell Lines: Three Examples

4.4.1 Establishment of Chordoma Cell Lines

Chordomas are rare malignant tumors that develop from embryonic remnants of the notochord and arise only in the midline from the clivus to the sacrum. Surgery followed by radiotherapy is the standard treatment. As chordomas are resistant to standard chemotherapy, the establishment of more chordoma cell lines is urgently needed to find alternative treatment options [6, 8, 9, 13]. Chordomas and resulting cell lines are characterized according to their morphology, expressed by a variety of lysosomes, vacuoles, and typical physaliferous bodies. Immunohistochemistry shows brachyury and cytokeratin 8 positivity and reveals a slow growth kinetic. It is very tricky to establish chordoma cell lines, due to tumor cells which are very slow growing and being surrounded by other fast-growing cells, such as stroma and immune cells. To get rid of fibroblasts, selective adhesion and detachment techniques can be used [10, 23].

4.4.1.1 Establishment of Sacral Chordoma Cell Line MUG-Chor1

To establish a chordoma cell line, tumor tissue was obtained immediately after surgery. Following mechanical disaggregation of the tumor tissue into approximately 1–2 mm^3 pieces, the mixture of cultures was carried out in fractions. Fraction I contained mechanically dissociated tumor cells. Fraction II comprised a cell fraction after the tumor pieces were treated enzymatically, in detail with 200 µL collagenase for 30 min at 37 °C, and fraction III included only the tumor pieces, pressed down with a cover glass (Fig. 4.4), to facilitate the growth of the cells to the cell culture flask. All fractions were cultured in Iscove/RPMI 4:1 containing 10% fetal bovine serum, 1% insulin-transferrin-sodium selenite (ITS), 2 mM glutamine, and 1% penicillin/streptomycin. Incubation was carried out at 37 °C in a humidified atmosphere of 5% CO_2. The chordoma cells grow at a pH of 7.4. Following a culture period of 4 months and a passage number of 5, the cells underwent a crisis. FBS concentration from 10% to 20% and the ITS concentration from 1% to 5% were increased. After applying these conditions for 1 week, the initial medium was used again. After that crisis, the cells grew consistently and showed a doubling time of approximately 7–10 days. Culture medium was changed twice a week, and splitting of the cell culture was performed every 10 days at a confluence of 70–80%. All cell cultures were periodically checked for mycoplasma by PCR [24].

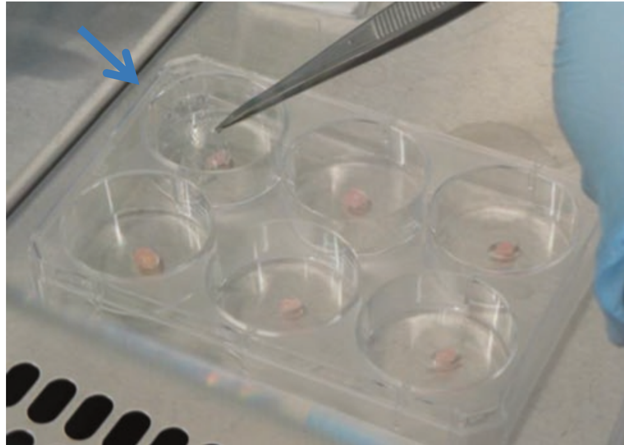

Fig. 4.4 The sterile cover glass (*blue arrow*) is pressed down on the tumor tissue with a forceps

4

4.4.1.2 Establishment of Clival Chordoma Cell Line MUG-CC1

A further problem in the establishment of tumor cell lines is to maintain the vitality of cells during tumor surgery. Various surgery techniques, for example, resectoscopy, can have an influence on the viability of the cells; also clival chordoma cells are very difficult to establish due to their slow growth and the location of the tumor. Therefore a particularly careful surgery method was chosen. The interdisciplinary skull base unit of the Medical University of Graz has contributed substantially to the development of four-handed transnasal transsphenoidal purely endoscopic approaches. The focus lies in careful tissue preservation and procurement of sufficient cell material as a prerequisite for optimal cell culturing. After appropriate access to the clival region and tumor exposure, preliminary tumor debulking was performed. The specific advantage of this surgical approach lies in the direct visualization of the lesion and the opportunity to obtain non-contaminated (by blood, mucus, mucosa, etc.) and structurally intact tumor tissue from the vital tumor mass [11]. The primary cells for the establishment of the cell line were obtained from a 72-year-old male patient who was referred to the Department of Neurosurgery because of a right-sided abducent nerve palsy causing double vision, vertigo, and cephalea. After mechanical dissociation, the cells were in vital conditions but expressed a slow growth [11].

If cells of interest show a very slow growth behavior, there is a risk that other cells will consume the media and displace the tumor cells. To separate the cells immediately from the culture start is also tricky, because they require growth factors and cytokine from surrounding cells. During the establishment of the cell line MUG-CC1, there was an overgrowth of B-cells, and tumor cells began to die, either because of medium consumption of fast-growing B-cells or of disharmony of both cell lines (Fig. 4.5).

Suspension cells are easy to separate from adherent cells. Nevertheless the right separation time point is important; otherwise the tumor culture will be lost. At that time point, when there are suspension and adherent cells together in culture, a fatal error can happen; in some cases it has been wrongly thought that the spontaneously immortalized suspensions cells might be tumor cells. Therefore the characterization of the suspension cells is very important.

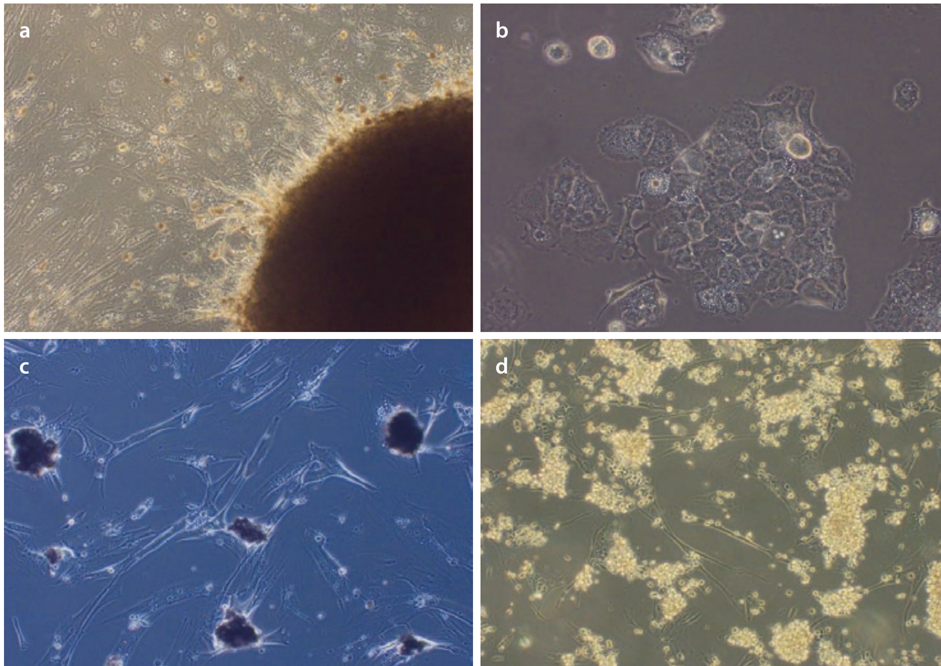

Fig. 4.5 Establishment of MUG-CC1 and outgrowth of tumor cells and surrounding cells from a tumor piece **a**, morphological typical chordoma cells with large vacuoles and physaliferous bodies separated from all other cells **b**, chordoma cells and suspension cells (brown cells on the top of the tumor cells) **c**, overgrowth of lymphoblastoid cells and displacement of tumor cells **d**

In the characterization of lymphoblastoid cell lines (LCL) during the establishment of MUG-CC1 by morphology, lymphoblastoid cells grew in a typically rosette morphology in suspension clusters [15], along with single cells. Flow cytometry analyses were positive for B-cell markers (CD19), while markers for T cells (CD3) and natural killer cells (CD56) were absent. DNA content of the cells can also be useful for characterization, while the tumor cells showed mostly an aneuploid DNA content and lymphoblastoid cells a diploid DNA profile. Copy number profiling showed a balanced profile, suggesting a non-tumorigenic cell line. Strong positivity for the EB2 (BMLf1) gene of EBV (TC 70 and TC 72) was detected in MUG-CC1-LCL, indicates an EBV transfection, and explains the immortal state [11].

4.4.2 Establishment of Aggressive Growing Melanoma Cell Line: MUG-Mel2

The cell line MUG-Mel2 was obtained from a fresh specimen of a cutaneous metastasis of a 48-year-old male patient with a cutaneous primary, ulcerated melanoma (8.5 mm thickness). The genetic analysis of the primary tumor and cutaneous metastasis revealed a mutation in NRAS Q61R; no mutations in BRAF or c-kit were detected. The tumor

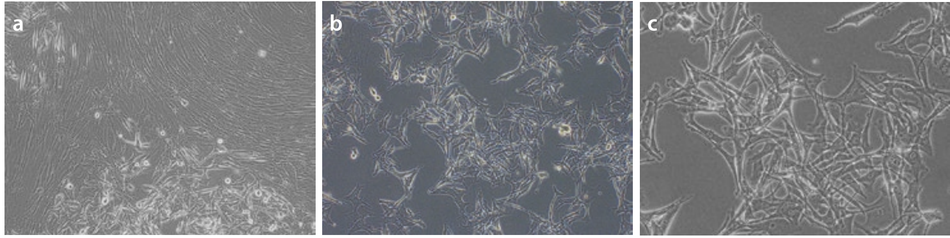

Fig. 4.6 Establishment of a fast-growing tumor cell line. Outgrowth of melanoma tumor cells surrounded by stroma cells **a**; MUG-Mel2 cell line after 10 days of cultivation, magnification ×10 **b**; MUG-Mel2 cell line in a higher magnification ×20 **c**

and the established cell line expressed melanoma markers such as Melan-A, HMB-45, S100, and tyrosinase. The tumor tissue (cutaneous metastasis) was obtained immediately after surgery; after mechanical disaggregation of the tumor tissue into approximately 1–2 mm^3 pieces, cells were cultured in RPMI (Life Technologies, Carlsbad, CA) containing 10% FBS, 2 mM L-glutamine, and 1% penicillin/streptomycin. Melanoma cells grew at a pH of 7.4. Cells were grown to 80% confluence and detached from the flasks with Accutase. Especially during culture start, cells should be kept in a very high density to encourage cell-cell contact and cell growth. Incubation of all cells was carried out at 37 °C in a humidified atmosphere of 5% CO_2. All cells were periodically checked for mycoplasma by PCR. Figure 4.6 shows prominent cells with triangular dendritic morphology as typically seen in melanoma and surrounding fibroblast-like cells, from culture start. Due to the rapid growth of the melanoma cells, other cells like fibroblasts were displaced. After 10 days, exclusively melanoma cells were further cultured (Fig. 4.6b, c), and all melanoma markers mentioned above were able to be kept in culture.

4.5 Quality Check

4.5.1 Recording and Freezing

The most important task during cell line establishment is to keep a complete documentation of the cell culture work. Each lab should create their suitable labeling modality. For each cell line, a separate aliquoted medium flask should be used. Inscription is very important; also expiry dates have to be respected. Morphological observation and pictures during culturing start is a must, as well as cryopreservation in regular intervals, to have a frozen stock of different passages available. For cryopreservation a mixture of 90% FBS and 10% DMSO is recommended. Don't freeze to less cells; if you have a small amount of cells, use a smaller cryo-vial with a lower volume.

4.5.2 Contaminations

There is a variety of contaminations in cell culture, divided into two main areas: microbial organisms and cell-cell contaminations. The more you work with a culture, the greater the risk of contamination. Handle only one cell line at one time, especially when working with primary cells. Aseptic techniques are required to prevent microbial contaminations.

4.5.2.1 Microbial Contaminations

Major contaminations in cell culture are bacteria, mycoplasma, fungi, yeasts, and viruses. Contamination source can be poor aseptic techniques, media additives, or the tumor tissue (e.g., mycoplasma). It is essential to monitor and test for contamination. Easy to detect are bacteria (increased turbidity or cloudiness, fine granules, movement), yeasts (small oval-shaped organism in short chains), and fungi (thin filamentous mycelia), whereas mycoplasma and viruses are more difficult to detect. Mycoplasmas are small prokaryotes and their presence cannot be observed by microscopy. The consequence of mycoplasma contamination can include growth rate effect [25], chromosome alterations [1, 18] nucleic acid and amino acid synthesis, as well as membrane alterations [17, 22, 27, 28]. A lot of different mycoplasma detections have been described with advantage and disadvantage in respect to cost, time, reliability, specificity, and sensitivity [16]. PCR and ELISA technologies present a very sensitive, specific, and rapid option to detect mycoplasma while covering a broad mycoplasma panel. The most difficult contamination to detect is virus contamination, but unless they are cytopathic, they may have little effect on their host cells [16].

4.5.2.2 Cell-Cell Contamination

"False" cell lines – cross contaminations of cells in culture was highlighted more than 30 years ago by demonstration that cell lines are contaminated by HeLa cells [20]. Especially when working with primary cells, cell-cell contaminations can easily occur. Causes of cell misidentifications can be human errors (mislabeling, unsterile working conditions, cross contamination) or an undesired result of the used techniques (use of feeder layers or xenografting) [21]. The more you work with a culture, the greater the risk of contamination. Handle only one cell line at one time and make sure that each cell line has its own media, trypsin, and PBS. Especially adherent cells can be kept apart due to their morphology, but the morphology of primary cells is still unclear. To be sure to work with the right cells, DNA profiling should be done. STR DNA profiling technology is used for routine identification (authentication) of human cell lines, stem cells, and tissues. STR analyses refer to short tandem repeat DNA, to examine individual areas in DNA. Difference for certain DNA regions can be used to distinguish between individuals. However, a human STR analysis is limited to the identification of species. This means that contamination with cell lines from another species, such as mice or rats, cannot be identified. The presence of nonhuman DNA remains undetected in human-based STR analyses. Animal species can be detected by isoenzyme analysis. Even during the establishment of cell lines, the cross contamination risk is very high! Therefore never treat primary cell culture and continuous cell lines at the same time. False cell lines cost time and money and not to be underestimated can cause incorrect publications.

Take-Home Message

Ten Golden Rules to Be Successful in the Establishment of Tumor Cell Lines

1. Well-preserved tumor tissue with high amounts of tumor cells.
2. Do not discard anything during cultivation.
3. Choose and track carefully cells of interest.
4. Regarding of media osmolality.
5. Tumor cells which grow adherent in the body will keep this ability and will grow adherent in cell culture!
6. Don't forget to check quality regularly.
7. Handle only one primary cell or cell line at one time.
8. Carefully and correctly labeling.
9. Produce frozen stocks.
10. Be patient.

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The Third Dimension in Cell Culture: From 2D to 3D Culture Formats

Verena Charwat and Dominik Egger

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What You Will Learn in This Chapter

Traditional two-dimensional (2D) cell culture does not represent the natural environment cells experience in their respective tissue or organ. In contrast, the cultivation of cells in a three-dimensional (3D) environment can help to generate a physiologic environment. This chapter describes the fundamental biological and technological differences of 2D to 3D cultivation and gives an overview on important 3D cell culture techniques. Different approaches for matrix-free and matrix-based cell culture as well as less conventional 3D cell culture approaches are described and discussed. Furthermore, the application of 3D cell culture in the field of tissue engineering and in vitro test models is presented.

5.1 Fundamentals of 3D Cell Cultures

5.1.1 Standard Cell Culture

Cultivation of cells from multicellular organisms (plants, animals, human) outside of the original organism has a long tradition with early examples dating back to the beginning of the twentieth century (details can be found in ► Chap. 1). Over time, different methods for ex vivo or in vitro cell cultivation have been developed and optimized. Today, mammalian cell cultivation is relatively well characterized, and the main aspects are standardized despite the diversity of specialized procedures [18]. The focus of this chapter will be on adherently growing cells since the concept of three-dimensional (3D) cultivation is of low relevance for suspension cultures. Typical parameters for routine cell cultivation as performed in most laboratories around the globe (in the following referred to as *standard cell culture*) involves the following aspects [18]: Cell culture incubators provide a favorable atmosphere for cell growth. They keep the temperature at a constant 37 °C. Typically, this temperature is used even if the body temperature of the respective species or tissue is different. A carbon dioxide (CO_2) concentration of, e.g., 5% is maintained in the gas phase. This is important to keep a favorable pH value (around pH 7.4) since the majority of culture media contain a carbonate-based buffer system, which interacts with the CO_2. Furthermore, the atmosphere in the incubator is humidified to minimize evaporation. The culture medium varies for different cell types, but traditionally a chemically defined basal medium containing glucose, inorganic salts, amino acids, vitamins, the carbonate-based buffer system, and often the pH indicator phenol red is used. Serum (mainly of bovine origin) is still the most common supplement and serves as a rich but ill-defined source of growth factors, hormones, and attachment factors. Sometimes antibiotics and/or antimycotics are added to prevent microbial contamination. The cells are maintained in plastic dishes typically made of polystyrene with surface modifications to enhance cell attachment. The most commonly used cell culture dishes are petri dishes, flasks, and multiwell plates. Cells grow on the flat bottom of the culture dish submerged in medium. The standard procedure for cell splitting involves enzymatic or mechanical detachment from the growth surface once 60–100% of the area is covered with cells. After harvest, a portion of the cells is transferred to a fresh dish for further cultivation. Since the cells grow on a flat surface and typically do not form more than one cell layer, *standard cell culture* is considered a two-dimensional (2D) cell culture technique.

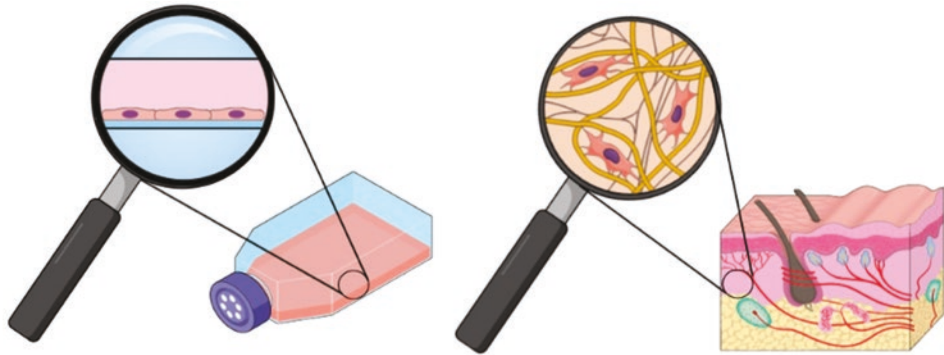
Over time, 2D *standard cell culture* has proven to be extremely useful for a wide range of applications from basic research to drug development and personalized medicine. Cell

cultivation is a very effective tool to study biological processes on a cellular level. It provides stable and reproducible conditions and facilitates assay standardization. The protocols can be easily exchanged between different laboratories. A wide range of disposable cell culture lab ware and supplementary equipment for handling and analysis (e.g., plate readers) is available from various suppliers. Most of the knowledge on cell biology we have today would have been near impossible to gain without experiments based on *standard cell culture* techniques.

5.1.2 Biological Differences Between 2D and 3D Cellular Microenvironments

Despite the undisputable value of *standard cell cultivation*, it also has some major drawbacks. The main limitation of *standard cell cultures* lies in the fact that they provide an environment, which is fundamentally different from the conditions found in actual living tissues. This includes nonphysiological medium composition (e.g., antibiotics, phenol red, high glucose concentrations) and excessive amounts of culture medium (the liquid/cell ratio is much lower in vivo). Furthermore, oxygen concentrations of ~21% are usually present in the gas phase of cell culture incubators, which is much higher than typical oxygen concentrations in body tissues. Traditional cell culture technique also fails to provide relevant mechanical forces such as compression, strain, and shear stress, which are always present in a living organism. Another limitation is the cultivation of (mostly) only one cell type in each culture dish (monoculture), while healthy tissues are composed of multiple cell types that strongly interact with each other. Many of these aspects are addressed in detail on other chapters of this textbook. The most striking difference – which is the focus of this chapter – however, is the fact that tissues are complex three-dimensional constructs as opposed to the cellular monolayers found in standard cell cultures. The implications of this difference are manifold and have been elucidated in several scientific review articles [1, 8, 16]. ◘ Figure 5.1 provides an overview of the most relevant biological differences between 2D and 3D cell cultures.

Most evidently, cell spreading is limited to two dimensions (x and y axis) in 2D formats, while a third dimension (z-axis; height) is added in 3D formats. Therefore surface area is a main limiting factor for cell growth in 2D cultures. Once the entire surface area is covered with cells, a passaging step has to be performed: the cells are harvested by disrupting cell-substrate and cell-cell adhesion, and a smaller fraction of the cells is transferred to a fresh culture dish. By contrast in a 3D environment, cell growth is usually not limited by the surface area, which is typically very large (e.g., in porous scaffolds). Instead, cell spreading and migration can be hindered sterically by the biomaterial. Mammalian cells typically measure 10–25 μm in diameter with a nucleus of ~10 μm diameter. Although cells are flexible and can adapt their shape to the environment and according to the respective cell type, they will be excluded from pores that are significantly smaller than their nucleus. For example, membranes with pore sizes of 0.3–3 μm are often used in drug testing to allow drug diffusion while preventing cell migration. However, the main limiting factor for 3D cell cultures is the diffusion-based distribution of oxygen, nutrients, signaling molecules, and waste products. In a compact 3D culture format (e.g., spheroids), cell proliferation is limited to a maximum of a few hundred micrometers from the surrounding medium. Therefore 3D cell cultures are often limited in size. To overcome this



2D cell cultures

- Soluble gradients absent
- Forced apical-basal polarity
- Continuous layer of matrix
- Unconstrained spreading and migration in x-y
- Adhesions restricted to x-y plane
- High substrate stiffness

3D cell cultures

- Soluble gradients present
- No prescribed polarity
- Discrete matrix fibrils
- Spreading and migration sterically hindered
- Adhesions distributed in all 3 dimensions
- Low substrate stiffness

Fig. 5.1 Schematic representation of differences between 2D and 3D cell cultures. Note that the full complexity of actual living tissue is still much greater than what can be usually mimicked in a 3D cell culture model. (Figure content based on [1])

limitation, larger 3D cell cultures require some sort of artificial "blood vessels." These could be anything from real blood vessels to predefined channels, interconnected pores of a scaffold, or diffusible hydrogels.

While the diffusion-based supply of 3D cell cultures provides some technological challenges for larger tissue constructs, it is also a highly relevant factor for providing in vivo-like conditions. Gradients of oxygen, nutrients, and signaling molecules are present in any living tissue. They play important roles in physiological and pathological processes. The best known example is solid tumors. A tumor is characterized by excessive cell proliferation. Consequently, the tissue volume at the site of disease increases until areas of the tumor or too far away from the closest blood vessel and cannot be supplied properly anymore. As a consequence, the inner core of the tumor starts to undergo necrosis. At the same time, however, the gradient of reduced oxygen and nutrients prompts the cells to release certain signaling molecules that induce blood vessel formation. By this mechanism the tumor can grow further and establishes its own supply system. Obviously, such complex processes cannot be studied in 2D cultures where virtually no gradients are present and each cell is exposed to essentially the same conditions. Similarly, drug tests – especially for cancer treatment – can provide more relevant results in 3D cultures as protective effects within the 3D cell aggregate are recapitulated. Consequently, today solid tumor research is the number one field of application for 3D cell cultures.

Another fundamental difference between 2D and 3D cell cultures is the properties of the substrate for cell adhesion. In standard culture dishes, the cells are facing a continuous, flat surface. Each cell will attach to this substrate with its bottom side, while the cells' top side is facing into the culture medium. Direct cell-cell contacts are limited to the thin

outer circumference of each cell. This configuration forces a basal-apical polarity on the cells. While such a polarity is physiological for cell types that form (mono-)layers in vivo, such as endothelial and epithelial cells, it is an unnatural configuration for most other cell types. Forced polarity can lead to an altered cellular phenotype and eventually bias experimental results. By contrast, 3D cultures do not prescribe cellular polarity but allow cell adhesion in all directions. Additionally, also cell-cell junctions can be formed in all dimensions. Notably, absence of forced polarity provides more in vivo-like conditions for most cell types. However, it might not constitute an ideal environment for the before mentioned cell types with native polarity. Generally, the culture format has to be chosen carefully with regard to cell type and intended application. No single cell culture approach (neither 2D nor 3D) will be ideal for all possible application scenarios.

Besides prescribing cellular polarity, 2D cell culture dishes also differ from natural tissues with regard to substrate stiffness, surface structure, and surface chemistry. Cell culture vessels are mostly made from cell culture-treated polystyrene (PS) or – less frequently – glass. Both materials have a stiffness that is orders of magnitude higher than that of most tissues. While the Young's modulus of PS and glass is in the range of GPa, a physiologically relevant range of 0.1–300 kPa has been described [15]. A large number of studies have documented several distinct effects of substrate stiffness on cell behavior including differentiation, growth, and drug response [12, 19, 24]. In some cases cell culture dishes are coated to reduce stiffness and provide physiological adhesion sites. The most commonly used coatings are compounds of the extracellular matrix (ECM) such as collagens (or its derivative gelatin), fibronectin, or vitronectin. In 3D cultures the same materials are often used in the form of hydrogels or for coating of solid, porous scaffolds. By contrast to PS or glass substrates, 3D casted matrices provide discrete fibrils as opposed to the continuous matrix layer found in 2D. Similar to surface stiffness, also the surface topography (spacing and geometry of adhesion points) significantly impacts cell phenotypes such as adhesion and differentiation [22]. Generally, 3D culture formats allow a much higher degree of flexibility with regard to optimization of physical and chemical properties of the cellular microenvironment. A considerable number of research groups have specialized on identifying and processing biomaterials for specific applications, and many products are already commercially available. An extensive discussion of different biomaterials and their implications for cell cultures is provided in ► Chap. 6. However, it needs to be noted at this point that a significant portion of 3D culture formats do not employ any biomaterial at all. These matrix-free culture systems rely on the ability of cells to interconnect, form cellular aggregates, and produce their own ECM. The best know example for this type of culture is spheroid cultures, which will be addressed in greater detail in the following sections. Fluorescence images of a 2D cell culture and several 3D culture formats of mesenchymal stem cells are provided in ◘ Fig. 5.2.

5.1.3 Technological Differences Between 2D and 3D Cultures

Although 3D cell cultures provide advantages with regard to increased physiological relevance, they are still not routinely used in many laboratories. Even in facilities that use 3D cell cultures, these are typically employed only for the final stage of an experiment or application, while routine handling and expansion is performed in 2D cultures. Overall, 2D cell culture still remains the standard cell culture format. The reason lies mainly in technological challenges associated with 3D cell cultivation. Compared to 2D cell cultivation, 3D cultures have been developed relatively recently. Most of the cell culture protocols, reagents,

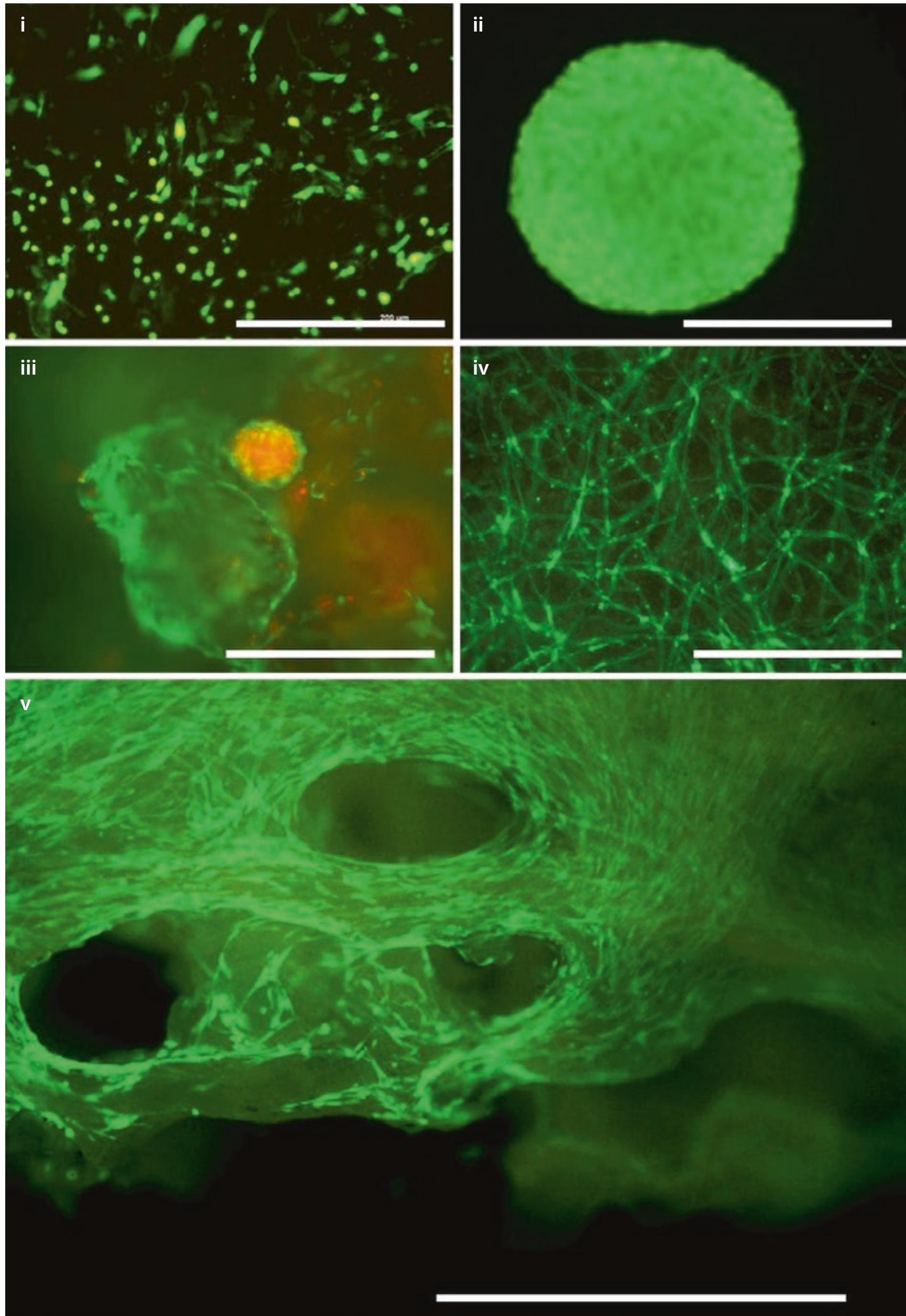


Fig. 5.2 Fluorescence microscopic images of mesenchymal stem cells in different culture formats after Calcein AM (*green*) and PI (*red*) live/dead staining: (**i**) 2D culture, (**ii**) matrix-free spheroid, (**iii**) embedded in nano-fibrillar cellulose hydrogel, (**iv**) co-culture with GFP-HUVEC in fibrin hydrogel (no live/dead staining), (**v**) on decellularized bone matrix. Scale bars represent 500 µm unless indicated differently

and materials we know today have been developed for standard cell cultivation. Unfortunately, in many cases they cannot be directly applied to 3D cell cultivation but require – sometimes extensive – adaptation and optimization. In some cases even completely different strategies are required for 3D cell cultures. The fact that 3D cultivation lacks behind 2D culture with regard to standardized protocols limits its general acceptance in research and industry. Furthermore, the 3D cell culture formats available today are typically more expensive and time-consuming than standard cell cultivation. Key goals for future research and development will have to include streamlining of protocols, identification of most suitable cell lines and primary cells, and optimization of matrix materials and consumables. Especially for applications in the pharmaceutical industry, sample handling techniques and equipment need to be compatible with high-throughput screening.

Analysis of 3D cell cultures presents a special technological challenge. Reliable, accurate, and reproducible analysis methods are key to any scientific experiment. Similarly to handling protocols and materials, also most of the currently available analytical techniques have been developed for 2D standard cell cultures. Initially, those methods have been directly applied also for 3D cultures, but results were often less than unsatisfactory. The main differences between 2D and 3D cultures that render analysis fundamentally different are listed in ◘ Table 5.1. Microscopy – including fluorescence microscopy – is the most widely used method for cell culture analysis. Visual inspection is essential for routine checks including morphology, confluency, absence of microorganism contamination, and cell counting. Live-cell and end-point imaging techniques constitute an integral part of most cell culture experiments. While microscopy allows close observation of 2D cell layers, multiple challenges arise for 3D cultures. The main limitations are associated with opaque samples, low contrast of cells compared to most scaffolds, obscuring light scattering, strong background fluorescence, and sample thicknesses greater than the microscope's working distance. Often only the outer cell layers can be evaluated microscopically, while the core of the sample or any structures more than a few hundred micrometers from the surface cannot be observed, resulting in potentially misleading data. Ultimately wrong conclusions might be drawn from such ambiguous data. Over time various approaches have been established to improve imaging of 3D cell culture samples. For example, confocal microscopes are used for layer-by-layer scanning (Z-stack) of fluorescent samples

◘ **Table 5.1** Differences between 2D and 3D cultures regarding analytical approaches

	2D cultures	3D cultures
Cell distribution	Homogeneous	Inhomogeneous, sometimes aggregated
Light penetration	Homogeneous, low background	Depth-dependent absorption, autofluorescence, scattering
Reagent diffusion	Homogeneous	Limited, inhomogeneous
Secretion into supernatant	Homogeneous	Limited, inhomogeneous
Lysis	Usually complete	Often incomplete with standard methods
Cell harvest	Routine procedure, single cells	Often incomplete or impossible, risk of cell aggregates

followed by assembly of the images to a 3D representation. Tissue clearing approaches can be applied to reduce light scattering. However, they require fixated samples and therefore can be used only for end-point imaging. Other imaging approaches have been adapted from tissue analysis. These include histological slices, micro- or nano-computer tomography (µCT, nCT), and magnetic resonance imaging (MRI).

Besides imaging approaches, virtually all other common methods for cell culture analysis are also applied to 3D cultures. However, each of them faces their own challenges and need for adaptation and optimization (see also ◘ Table 5.1). In the simplest case, the culture supernatant is collected, and soluble factors such cytokines, enzymes, metabolites, and dissolved gas are identified and quantified. Examples of typical methods include ELISA tests, glucose/lactate measurements, and LC-MS. Additionally, some viability assays based on colorimetric or fluorometric identification of loss in membrane integrity (e.g., LDH release) also fall into this category. Since these assays rely on analysis of the supernatants instead of the cells, their quality is not directly affected by 3D cultures. However, it needs to be considered that supply within the 3D construct is diffusion limited. Drugs or other test substances will not reach all cells homogenously, and – even more importantly – secreted factors might not be transported into the supernatant effectively. The same phenomena of diffusion limitation and inhomogeneous supply are even more pronounced in non-lytic assays involving intracellular staining or substrate conversion. Examples are metabolic activity assays based on intracellular reduction (e.g., resazurin) and live/dead staining (e.g., Calcein AM/PI). Typically, the outer cell layers will contribute to the overall signal much more than the sample core. This limitation can be alleviated using lytic assays where the 3D construct is destroyed and cells are lysed to quantitatively release their cytosolic content. Prominent examples of detection methods include qPCR and Western blot. Although in lytic assays, theoretically each cell contributes equally to the final signal, protocols need to be optimized to ensure complete lysis of the entire sample.

5.2 Types of 3D Cell Cultures

5.2.1 Matrix-Free 3D Cell Cultures

Adherently growing cells feature three important capabilities that allow them not only to grow in standard cell culture dishes but also to effectively form three-dimensional assemblies: They can form connections between cells (cell-cell interaction) and between cells and a substrate material (cell-matrix interaction) and also produce their own substrate (ECM). So-called matrix-free 3D culture formats take advantage of these properties by depriving cells of their bottom substrate (e.g., polystyrene surface). Most adherently growing cell types cannot survive in suspension for extended time periods. To form vital cell-cell and cell-matrix interactions, they are forced to assemble into cell clusters. Eventually, the cell clusters become more compact as cells form stronger connections and produce ECM. A main advantage of matrix-free cultures is the absence of any foreign material, which might have unintended biological effects. Instead, each cell type produces its own native ECM.

Different approaches to prevent cell adhesion on a 2D substrate exist. The best known and most widely used method is *hanging drop cultivation*. Here the traditional cell culture setup is turned “upside down,” having a substrate layer on top and small drops of cell sus-

pension, typically 20–50 µL, hanging down. The cells sink to the bottom of the drop. As the air-liquid interface forms a natural barrier, the cells aggregate in the drop's center. Depending on the cell type, it typically takes 24 h to several days until a compact and stable *spheroid* has formed (see ◘ Fig. 5.2ii). A simple and inexpensive option is the use of petri dishes, where several drops of cell suspension are pipetted into the lid, which is then carefully flipped over in a single smooth motion. Several companies have also marketed specialized well plates to facilitate cell handling and increase throughput and reproducibility. Those systems are typically compatible with automated procedures used in industry.

A typical challenge associated with hanging drop cultures, especially when performed manually, is the retrieval and handling of the spheroids, which are typically 70–300 µm in diameter and often stick to pipette tips and other plastic surfaces. Hanging drop cultures are also highly susceptible to evaporation because of their small volume combined with very high surface to volume ratio. Therefore they need to be kept in a high humidity environment. Hanging drop culture dishes typically feature liquid reservoirs or humidifier pads soaked with sterile water or saline buffer. Additionally, the small medium volume needs to be replaced frequently, since nutrients are rapidly depleted.

Another simple approach for matrix-free cell cultivation employs *low-adhesion surfaces*. While standard cell culture dishes are surface treated to render them hydrophilic and attractive for cell adhesion, cell repellent surfaces can be used to force the cells into aggregation and spheroid formation. Low-adhesion surfaces can be achieved by chemical, physical, and topographical modifications – or a combination thereof. The main disadvantage of this method is low control over cell number per spheroid. Typically, a multiple spheroids of varying diameter will form in one dish. To overcome this limitation, so-called *U-bottom plates* can be used. These combine low-adhesion surfaces with the semispherical shape of hanging drops. The most common format is 96-well plates where – ideally – a single cell spheroid comprising all cells forms in the center of each well. This method requires larger media volumes compared to hanging drop cultures, which alleviates evaporation and nutrient depletion issues.

The main applications of spheroid cultures are drug screening and cancer research [14]. However, many other tissue-specific spheroids have also been developed and proven more physiological relevant compared to standard 2D cultures [6]. Regardless of the selected method, spheroid cultures are limited in size to several hundred micrometers in diameter due to diffusion-based supply. However, even at smaller size, necrotic cores might form (◘ Fig. 5.2iii). The optimal cell number, spheroid size, and duration of cultivation need to be assessed individually for each cell type and experiment. Some approaches such as integration of vascularization and dynamic cultivation strategies can mitigate but not completely resolve supply issues in spheroid cultures. More information on dynamic cell cultivation is provided in ▶ Chap. 10.

Spheroid cultures are by far the most commonly used matrix-free 3D cell culture format in research and industry. However, various other approaches have also been established. While no complete survey can be provided here, some interesting concepts are illustrated: *Cell sheet layering* is a bottom-up approach where cells are grown in standard culture until they form a dense and stable cell layer [21]. Then the complete cell layer is removed from the surface and placed on top of another cell layer in a different culture dish. Importantly, a special cell harvest procedure is required to preserve an intact cell layer instead of creating a cell suspension. "Smart" surfaces that change their properties from high to low adhesion upon external cues such as a change in temperature from 37 °C to room temperature have been successfully used for this purpose. The same procedure

can be repeated to stack several cell sheets; however as the layer thickness increases, cell supply via diffusion eventually becomes a limiting factor. In order to overcome this limitation, recent studies aim at incorporating vascularization [3].

Another heavily investigated technology for 3D cell cultures are *microfluidic systems*. A microfluidic system is a small, perfused device with channel dimensions in the range of micrometers. Microfluidic technology has been successfully used for various applications not only in cell biology but also in molecular biology, chemistry, physics, and other disciplines. The most obvious advantage of microfluidics for cell cultivation is the reduced consumption of reagents, which is especially relevant if rare cell types or expensive compounds are used. More importantly, however, microfluidics presents a technological toolbox that allows precise control over the cellular microenvironment including chamber geometry, surface chemistry, temporal and spatial reagent distribution, and mechanical stimuli. This enables multiplexing of screening assays and recreation of physiological niches [4]. Over the last decade, a vast number of microfluidic systems for 2D and 3D cell cultivation have been published. A current trend is the recreation of tissue, organ, and even complete body functions in a single microfluidic device [23]. In many cases physiologically relevant 3D cell assemblies can be achieved by tuning the chamber geometry without the need for additional matrix materials or scaffolds. This includes the fabrication of microchannels, micropillars, cell retention chambers, microvalves, and combinations of these elements. An early example is a liver model mimicking a single liver sinusoid. Hepatocytes were compactly loaded into a long, narrow chamber where they formed a 3D cell assembly. A special fenestrated wall and external medium perfusion allow for supply and maintenance of the entire tissue mimic. A recent example of a more complex microfluidic device for matrix-free 3D cell culture is a cardiac microphysiological system [13]. The cell culture chamber geometries were optimized to promote maturation of cardiomyocytes, which is typically incomplete in 2D cultures. A current trend that poses multiple biological and technological challenges is the combination of multiple microfluidic tissue models into a so-called body-on-a-chip system. If successful, this approach could revolutionize the drug development process.

5.2.2 Matrix-Based 3D Cell Cultures

Expanding the cellular in vitro environment by a third dimension contributes immensely to the generation of a physiologic environment. To extend cellular growth to the third dimension, supportive structures, also referred to as matrices or scaffolds, have been engineered from numerous materials. However the vast amount consist of ceramics (such as tricalcium phosphate or hydroxyapatite), synthetic polymers (such as polystyrene, poly-L-lactic acid, or polyglycolic acid), or natural polymers (such as collagen, alginate, or silk), each having different physicochemical properties, architecture, and biodegradability. Inherent material characteristics such as porosity, pore size and distribution, surface-to-volume ratio, mechanical characteristics, and surface chemistry have an influence on cellular behavior (see ► Chap. 6). Cell attachment, migration, proliferation, and differentiation were shown to be impacted by material characteristics. In return, 3D cultivation has a remarkable impact on the outcome of drug screening, cell shape, and cell-cell and cell-ECM interactions. Obviously, every material has its own advantages and disadvantages and must be therefore chosen to fit the respective biological requirements. Ceramics are porous structures with high stiffness and thus suitable for bone tissue engineering approaches, whereas softer, fibrous, or gel-like matrices are more suitable

for mimicking a skin, cartilage, or tendon environment. To combine the best of different materials, composites containing two or more materials are gaining interest. Recent advances in manufacturing technology of 3D materials, such as 3D printing, open up new possibilities to produce a completely defined architectural environment.

5.2.3 Other Types of 3D Cell Culture Formats

5.2.3.1 Tissue Slice Cultivation

Besides matrix-free or matrix-based 3D cell culture, also other formats are considered as 3D cell culture. Tissue pieces or thin slices of tissues or organs are mounted on a substrate to be cultivated outside their native organism [20]. This is thought to represent a more reliable model because the original architecture, cell-cell interactions, and cellular composition are maintained. Indeed, tissue slice cultivation was often shown to behave more physiologic than cell-line-derived tissue models. Typical tissues that have been cultivated this way comprise the liver, brain, intestine, and lung. Since tissue slice cultivation seems to be as close as possible to in vivo, it may provide an interesting alternative to animal testing to study, i.e., drug metabolism or toxicity. However, the cultivation period of tissue slices is comparatively short, and the availability of tissue slices from human origin limits a broad application of this technique.

5.2.3.2 Microcarrier (2½D)

Some culture formats cannot be classified into matrix-free and matrix-based techniques, because of their special properties. Microfluidic technology has already been presented as a technological platform that enables matrix-free as well as matrix-based 3D cell cultures (besides multiple other cultivation formats such as single cell and 2D cultures). Another prominent example is cell cultivation on *microcarriers*. Microcarriers are small beads, typically of spherical shape, with 100–300 μm diameter. They are made of cell culture compatible materials and feature surfaces that promote cell adhesion. Microcarriers have been used for over 40 years in production bioreactors (e.g., for antibodies or vaccines) if adherently growing cells are selected as production organism [2]. More recently, they have gained increasing importance for cell expansion for tissue engineering applications [11]. Microcarrier cultures can yield high cell numbers while preserving the characteristic cell morphology and phenotype. The use of microcarriers facilitates the adaptation of suspension culture protocols and equipment, such as stirred-tank bioreactors, for anchorage dependent cell types. In most cases the cells grow as a monolayer only on the outer surface of the microcarriers. Consequently, microcarriers are typically not true 3D culture formats. Because of their bent surface, microcarriers are sometimes referred to as 2½D culture systems.

5.2.3.3 Magnetic Nanoparticles

Nanoparticles are functional units with a diameter between 1 and 100 nm which can be fabricated from a broad spectrum of materials. They are of special scientific interest since they display often unexpected properties and thus have potential for applications in the fields of electronics, optics, physics, and medicine. Magnetic nanoparticles can be used to treat cells in order to control their spatial position in a magnetic field. The magnetic levitation method uses magnetic nanoparticles to levitate and concentrate cells without the need of a matrix to facilitate the formation of 3D aggregates with cell-cell interactions [7]. Also,

controlled 3D co-culturing of different cell types is possible with this technique allowing for the generation of more complex 3D cultures. Although, no external matrix is involved, magnetic levitation is different from other matrix-free culture formats: The magnetic nanoparticles constitute a foreign ingredient, which could potentially alter cell physiology.

5.2.3.4 Complex 3D Assemblies

To generate functional tissue, the interplay of multiple cell types in a complex matrix is often required. 3D assemblies are systems that incorporate multiple components, i.e., several cell types and/or matrices, in order to build a more complex and thus more functional tissue. For example, in the field of vascular tissue engineering, self-assembling cell sheets were developed. Smooth muscle cells and fibroblasts are cultivated in 2D for 30 days to produce a firm collagen-rich cell sheet which is subsequently rolled up on a mandrel to form a tubular structure. The inner lumen of this structure then undergoes endothelialization resulting in a matured functional vascular graft with equivalents to the three layers of a blood vessel (intima, media, and adventitia) [9]. Another complex 3D assembly was established to overcome thickness limitations in a 3D cardiac tissue graft. This cultivation system consists of triple-layered cardiac cell sheet on top of a collagen gel with embedded medium-perfused microchannels. Migration of endothelial cells from the cardiac tissue into the microchannels below was observed and resulted in a perfused tissue with a functional vasculature [17]. Furthermore, it was possible to expand the tissue by another three-layered cardiac cell sheet on top of the first one to build a comparatively thick tissue graft which was still sufficiently supplied with oxygen and nutrients.

5.3 Applications of 3D Cell Cultures

5.3.1 General Considerations

Applications of 3D cell cultures can be separated into two main categories: (a) in vitro test systems and (b) clinical application for tissue repair. Both share the common need for recapitulating human (or animal) physiology. The typical steps of a tissue engineering process are depicted in ◘ Fig. 5.3. First, the target cells need to be isolated from the respective organism. This can be either matured cells or multipotent stem cells which can be differentiated into the target cell type in vitro. Then, cells are expanded to yield a sufficient amount of cells for further operations. Subsequently, cells are combined with a biomaterial to generate a 3D cell-matrix construct which then can be either used in in vitro test models or for clinical applications.

5.3.2 Tissue Engineering

In the case of clinical application, the cells itself are the required product. The tissue-engineered 3D cell-matrix constructs are used to replace damaged or missing tissue. To function properly the engineered tissue needs to reconstitute the original tissue. For this, cells on the biomaterial need to be cultivated in an environment that represents the physiologic environment. Automated bioreactor systems are often used to generate and keep the necessary culture conditions, such as mechanical stimulation, during cultivation in order to produce functional tissue (see ► Chap. 10).

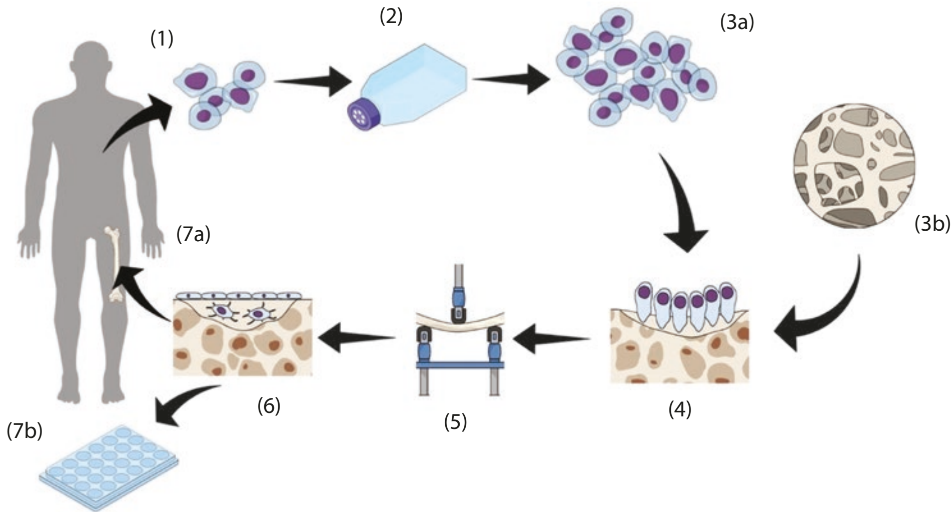


Fig. 5.3 Concept of the tissue engineering process: Cells are collected from a healthy donor site (**1**) and expanded in standard 2D culture (**2**). The propagated cells (**3a**) are combined with a biomaterial (**3b**) to create a 3D cell culture (**4**). Optional (mechanical) stimulation (**5**) can be applied to achieve tissue maturation. The final 3D cell culture construct (**6**) can then be used for clinical applications (**7a**) or as an in vitro test model (**7b**)

For clinical applications it is important that the entire cultivation process is performed according to the guidelines of the respective country. Good manufacturing practice (GMP) are widely used guidelines that comprise the minimum requirements a manufacturer must fulfill to meet a specific quality standard and to avoid risks for the consumer (patient). Furthermore, in Europe tissue-engineered constructs are considered as advanced therapy medicinal products (ATMPs) which are regulated by European Medicines Agency, while in the USA these kinds of products are regulated by the Food and Drug Administration (FDA). During clinical application good clinical practice (GCP) need to be considered.

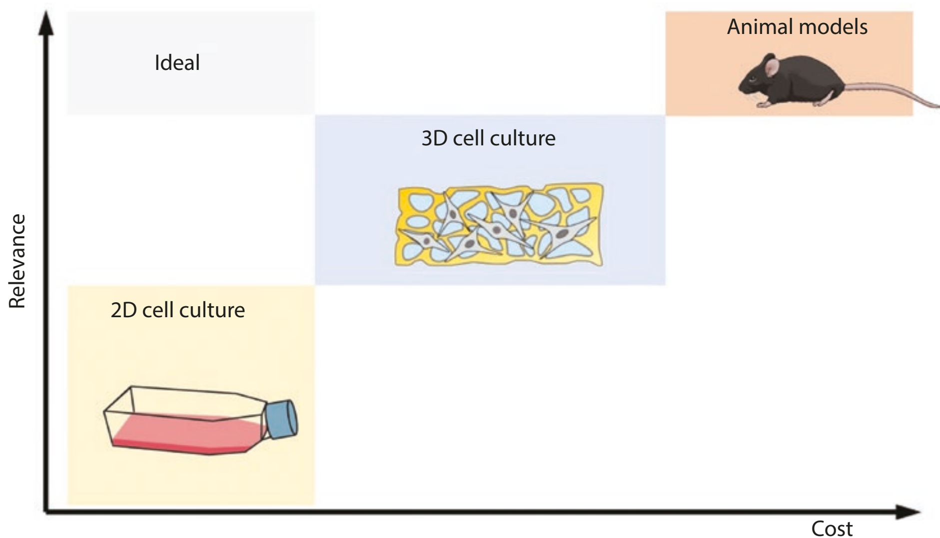
3D constructs for clinical application are always comprised of primary cells which can be either differentiated cells or stem cells. Embryonic stem cells (ESCs) are pluripotent meaning they can develop into almost every other cell type. Thus, ESCs seem to be the perfect source for any kind of tissue-engineered construct. However, since ESCs are derived from the inner cell mass of a blastocyst (an early-stage embryo), ethical concerns on the use of ESCs have ever been and still are present. Multipotent stem cells, such as mesenchymal stem cells (MSCs), can be isolated from many different adult tissues and thus are free of ethical concerns. Induced pluripotent stem cells (iPSCs) are reprogrammed somatic cells that can again develop into many different cell types and thus represent an interesting cell source. However, genetic and epigenetic aberrations of iPSCs have been observed, and thus iPSCs cannot be considered as safe so far. Therefore, MSCs are still considered as the most promising candidate for tissue engineering applications.

Although tissue engineering is more and more entering clinical applications, a major limitation is the construction of large-size tissues. Especially in the field of musculoskeletal engineering where large-size defects occur frequently, diffusion is not sufficient to supply the entire construct with oxygen and nutrients. Consequently, a necrotic core and thus non-functional tissues are observed. The dynamic cultivation, i.e., in perfusion

bioreactors, reduces diffusion gradients by active transport of the cultivation medium (see ► Chap. 10). Still, the effects of convection in dynamic cultivation are limited and thus a perfusable vessel-like network in a 3D cell-matrix construct is favorable. Different strategies have been developed to solve the problem of in vitro prevascularization. Vessel-like scaffolds of synthetic or natural origin can be seeded with vessel-forming cells such as endothelial cells and cultivated until the constructs are fully populated by them. Also, cell sheet technology can be used to generate monolayers of different cell types which are subsequently stacked to multilayer constructs including endothelial cells for the formation of blood vessels. Another approach incorporates spheroids composed of endothelial cells and/or undifferentiated MSCs into the tissue construct. The spheroids have been shown to develop a dense network of vessel-like structures. Furthermore, vascular channels and networks can be formed by molding a non-sacrificial material (i.e., hydrogels) around a sacrificial component (i.e., gelatin) which is then chemically dissolved, followed by seeding of endothelial cells. Also, 3D printing is heavily investigated as a possibility to generate off-the-shelf complex tissue with a functional vasculature [10].

5.3.3 Test Models

The most important application for 3D culture today is in vitro test models. Since 3D cultivation better represents the physiologic environment experienced by cells in the body, the cells react in a more physiologic way to external cues than in 2D cultivation. This is referred to as the "relevance" of a model. Today, animal models are accepted to have the highest relevance while at the same time being the most costly (see ◘ Fig. 5.4). 3D cell culture models are bridging the gap between conventional 2D cell culture models and animal models. Therefore, 3D cell cultivation is of enormous interest in the field of drug development as well as in the study of tissue development and diseases. Representative



◘ **Fig. 5.4** Relative relevance and costs of 2D and 3D cell culture and animal models

physiologic models of specific tissues such as the liver, intestine, or brain or pathophysiologic (disease) models and tumor models are needed to study drug metabolism and toxicity. In animal testing researchers must explain why there is no alternative to using animals for research. The underlying guidelines are called the "three R" rule: replace, reduce, and refine. Replace the use animals with an alternative approach or technique. Reduce the amount of animals needed for testing to a minimum while obtaining the same amount of information. Refine the experimental procedures to keep animal suffering to a minimum. Altogether animal testing is costly and ethically questionable, and the direct transfer of results to humans is often limited. Therefore companies with the need for animal testing are interested in highly relevant in vitro models that are less costly. 3D in vitro models do not only reduce animal testing but at the same time allow for high-throughput screening of different substances or conditions. A complex 3D in vitro model of a specific tissue is often referred to as an "organoid." They can be either derived from primary cells or ESCs and iPSCs, and for many tissues organoid models have already been developed [5].

A specific application of in vitro tissue models is the organ-on-a-chip technology which combines the 3D cultivation of different cell types with microfluidic systems to build a functional unit that mimics the behavior of an entire organ. Even 3D models do not represent the complex interactions between different tissues, such as tissue-to-tissue interface interactions or the transport of soluble cues such as growth factors. Organ-on-a-chip may therefore better simulate the actual in vivo situation than classic 3D models [23].

5.4 Summary and "Lessons Learned"

Traditional standard cell culture is very useful for a wide range of applications but is fundamentally different from physiologic conditions found in living tissues. In contrast, 3D cell cultivation enables for the generation of a more physiologic environment. Due to increased cell-cell and cell-matrix interactions as well as the possibility to migrate in all three dimensions, cells display a more physiologic behavior. Matrix-free approaches like spheroid or cell sheet cultivation foster cell-cell interactions and trigger the cells' own matrix production. With matrix-based approaches, the behavior of cells can be controlled via the architecture or functionalization of the matrix. Often 3D cultivation is carried out in a bioreactor to apply mechanical forces such as shear, pressure, or strain. 3D tissue constructs are already used for in vitro test models and in the field tissue engineering. However, limitations such as the development of a proper vasculature or the analysis of 3D constructs hamper broad applications.

Take-Home Messages

- The main aspects of so-called standard cell culture comprise temperature, composition of atmosphere, and medium.
- However, many aspects of standard cell culture do not represent physiologic conditions.
- The main differences between 2D and 3D cell culture are soluble gradients, polarity, discrete matrix fibrils, migration which is sterically hindered, adhesion possible in three dimensions, and lower substrate stiffness.
- Protocols for the analysis of 2D cannot be transferred to 3D without modification.
- Most approaches for 3D cell culture are either matrix-free or matrix-based.

- Mainly, 3D cell cultures are currently applied in tissue engineering processes and for in vitro test models.
- Animal models are costly and ethically questionable and the transfer to humans is limited.

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Biomaterials

Rainer Detsch, Julia Will, Jasmin Hum, Judith A. Roether, and Aldo R. Boccaccini

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What You Will Learn in This Chapter

Biomaterials are a special class of materials that have been engineered to take a form which, alone or as part of a complex system, is used to direct the course of any therapeutic or diagnostic procedure, by controlling interactions with components of living systems [1]. Furthermore, biomaterials can be classified as types of materials – be it natural or synthetic, alive or lifeless and usually made of multiple components – that interact with biological systems [2]. They are used either for therapeutic (treat, augment, repair or replace a tissue function) or for diagnostic (sensors, cancer models, animal test substitution) purposes.

This book chapter – *cells meet surface* – deals primarily with the most important aspect of biomaterials, i.e. their interactions with cells. More particularly, the chapter is focused on biocompatibility and characteristics for enhanced performance of permanent and non-permanent biomaterials, surface properties as well as tissue engineering and biofabrication.

6.1 Biocompatibility

There are several definitions for biocompatibility in the literature dependent on the different related disciplines including biology, material science, physics, chemistry and medicine. Overall, biocompatibility and also the opposite term "biotoxicity" can be viewed as a biomaterial property that involves physical, chemical, biological and medical aspects of a biomaterial. In a regulatory sense, biocompatibility is a materials property to determine the potential toxicity resulting from bodily contact with a material or medical device. Biocompatibility is an essential criterion for medical devices and encompasses both local and systemic reactions. That means that systemic reactions affect parts of the body away from the location of the implant or device [3]. The term biocompatibility itself is connected to three levels of the biological evaluation and addresses the biosafety of a medical device (◘ Fig. 6.1). Testing the biological behaviour of biomaterials always starts with cell culture trials (in vitro tests), followed by more comprehensive investigations on experimental animals (in vivo), and clinical studies are the final step of the evaluation process. With the increase of the investigation level, complexity and costs are also increasing.

The in vitro biocompatibility evaluation also termed "cytocompatibility testing" of materials or eluates is the first step in the assessment of biological behaviour of biomaterials and scaffolds: the standard EN 30993, part 5, gives guidelines to perform such tests [4]. In this norm, simple cell lines, like fibroblasts from mice (L929) or human osteoblast-like cells (MG-63), are suggested to evaluate cell proliferation, viability and morphology. In more advanced setups, primary cells or even stem cells can be used for more detailed understanding of cell-material interactions, and cell differentiation studies can be performed. However, it seems to be reasonable to exploit the target cell type l, for which the material has been intended for future implantation. Cell culture systems may be of value in testing the biocompatibility of prosthetic materials before they are introduced into clinical use. In recent years, in vitro methods for assaying biomaterials have gained importance owing to the growing concern over the use of animals for biomaterials testing.

Cells are generally more sensitive to toxic materials in vitro than in vivo tissue. Therefore, a material showing a moderate to high level of toxicity in vitro may result not particularly toxic for the tissue in vivo, while a material harmless to the cells, even in long-lasting assays, is likely to be inert also in vivo. However, the word biocompatibility refers also to the interaction of a living system or tissue with a finished medical device or

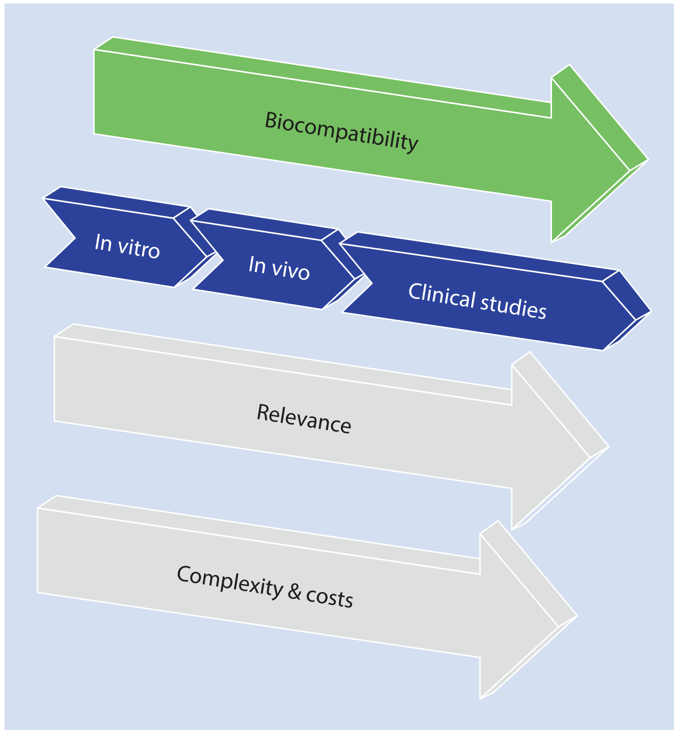


Fig. 6.1 Relationship between biocompatibility and its increase in relevance, complexity and costs

component material. The biological response of tissue cells to an implant can alternatively be assessed by tissue engineering and biofabrication approaches, which will be explained later in this chapter (► Sect. 6.4).

A medical device may comprise materials that are individually biocompatible; however, the composed device itself requires additional biocompatibility testing. For this reason, also macrophages, endothelial cells or even embryonic stem cells are used to determine the biocompatibility of a certain material. At last, in vitro cell culture test systems have to demonstrate that they are strong alternatives to animal tests.

6.2 Materials and Synthesis

6.2.1 Materials

A very large number of different biomaterials are available for biomedical applications. Generally, biomaterials can be divided into alloplastic (synthetic) and biological materials as illustrated in Fig. 6.2. Alloplastic materials can be further divided into different groups mainly ceramics, metals, polymers and composites of the former, whereas a biological material is living tissue from the same person (autograft), same species (autograft) or another species (xenograft).

Polymeric materials (e.g. PE, PMMA, PVC, etc.) are used either due to their flexibility or used for low-friction articulating surfaces. Natural polymers, e.g. collagen, starch or

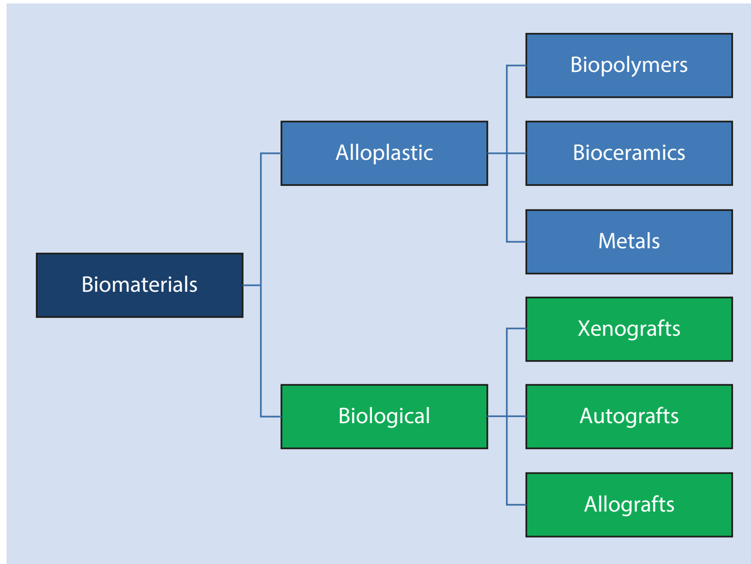


Fig. 6.2 Classification of biomaterials into alloplastic and biological and their subdivisions. With all these materials, several combinations for composites are possible [5]

fibrin, are also used as biomaterials having the advantage of being highly biocompatible and bioactive and their ability to undergo biodegradation. Ceramic materials (e.g. zirconia, alumina) are characterized by their high biocompatibility, hardness and wear resistance and are used in applications such as articulating surfaces in joints. Metals (e.g. titanium, Ti alloys Ti-6Al-4V, cobalt alloys or stainless steel) typically possess high tensile, high fatigue and high yield strengths and are therefore used for load-bearing applications such as bone and joint replacements. Composites with different combinations of the materials outlined above can be tailored to the properties required; they offer a variety of advantages in comparison with homogeneous materials as the drawbacks of each individual material can be minimized. It is important for biomaterials that each constituent of the composite must be biocompatible. Examples of composites currently used in biomaterial applications include the following: dental filling composites, bone particle or carbon fibre-reinforced methyl methacrylate bone cement and ultrahigh-molecular-weight polyethylene and porous surface orthopaedic implants (Fig. 6.3).

Synthetic biomaterials (ceramics, metals, polymers and composites) are prepared using a large variety of different processing methods. There are several industrial processing methods for producing synthetic biomaterials; however in this book chapter, only laboratory-scale technologies are presented.

6.2.2 Preparation

Polymer synthesis can be described as the process of covalently bonding monomers (small molecules) to form a polymer chain or network of repeating structural units (also termed "macromolecules"). The formation of polymers can be achieved by different methods, including polymerization, polyaddition or polycondensation. Natural polymers can be harvested from plants, animals and humans. Technical polymers are generally produced synthetically, whereby a variety of different techniques (injection moulding, blow casting,

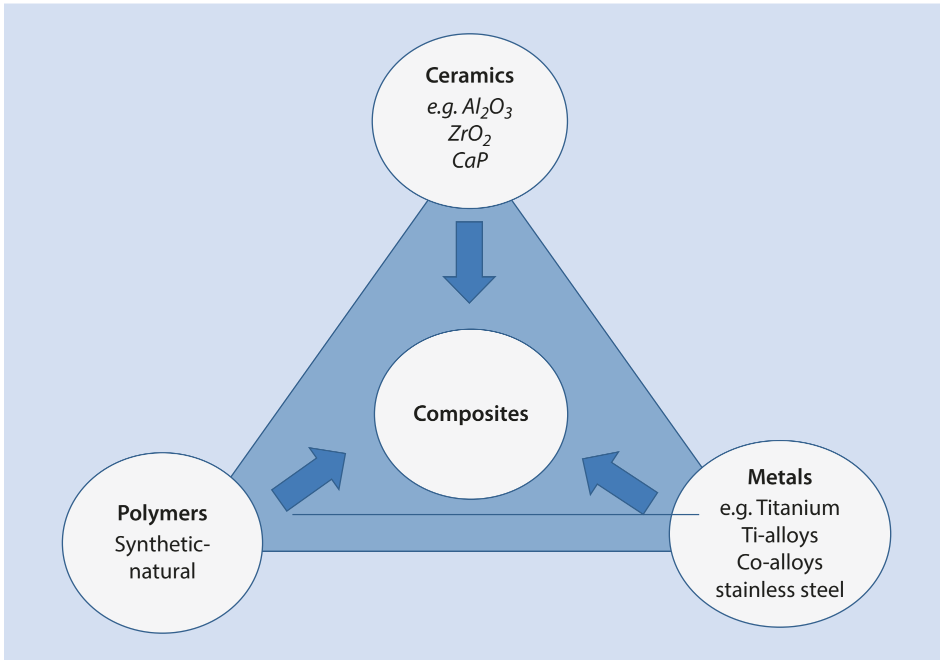


Fig. 6.3 Composite biomaterials are formed by combination of polymers, ceramics and metals, e.g. dental filling composites, bone particle or carbon fibre-reinforced methyl methacrylate bone cement and ultrahigh-molecular-weight polyethylene

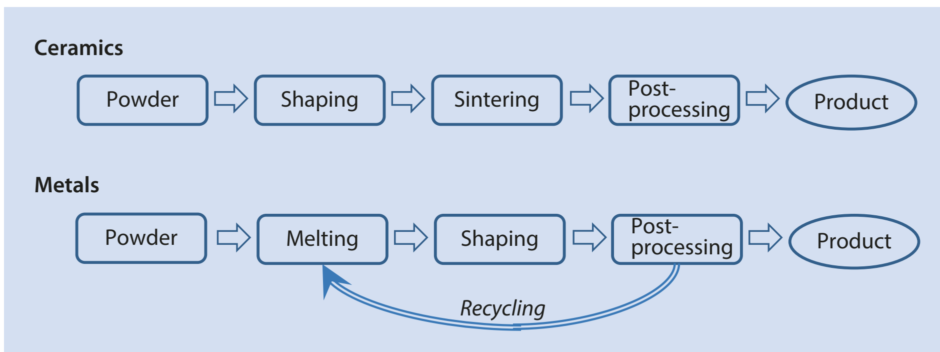


Fig. 6.4 Flow charts of the processing routes for ceramics and metals

extrusion, thermoforming, etc.) can be applied. Many processing techniques used for the synthetic polymers have also been applied to natural polymers.

Ceramics (e.g. Al_2O_3) are fabricated starting from a raw ceramic powder which can be found in nature or produced synthetically, followed by different forming processes (e.g. slip casting or dry pressing). A treatment at high temperature (sintering) converts the porous green body into a dense solid. This process is connected with a volume change. Afterwards, the ceramic can be post-processed via polishing. Metals require a melting process of the raw material (e.g. iron ore), after which it is cast into the desired form. Metals can be recycled after the post-processing step. Figure 6.4 illustrates simplified flow charts for the processing of ceramics and metals.

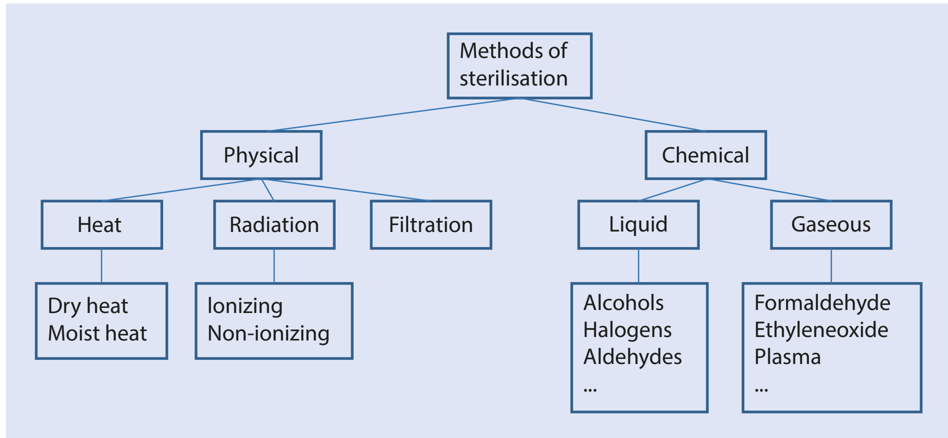


Fig. 6.5 Different physical and chemical methods to sterilize biomaterials

6.2.3 Sterilization

Sterilization is a process whereby harmful substances and living microorganisms (e.g. bacteria) are eliminated through the use of physical, chemical and physicochemical means (e.g. high temperature, intense radiation, concentrated toxic chemicals). The sterilization of a biomaterial is important for its success.

Biomaterials must be sterilized after processing and prior to packaging, so that their sterility can be maintained until the point of use. Sterilization depends on the contact of the sterilizing agent (e.g. dry heat, chemical solutions, steam, ethylene oxide, formaldehyde gas, hydrogen peroxide plasma, ionization radiation, etc.) with all surfaces of the item to be sterilized. The selection of the sterilization method primarily depends on the material to be sterilized, as the process should not induce a change of material properties. Whereas ceramics and metals are not so sensitive towards sterilization agents, for polymers a change of material properties, like crystallinity or elasticity, can incur.

The method of choice in most labs is autoclaving using pressurized steam, whereas in industry sterilization with gamma radiation is the most commonly used source for sterilization. The advantage of the latter process is the possibility to sterilize materials, which are already in their outer packaging (Fig. 6.5).

In this context it should be mentioned that UV radiation and alcohol solutions do not generate sterile conditions, as these are disinfection methods.

6.3 Material Characteristics

Cell-material interactions are a complex process and play an essential role for the integrity of biomaterials in the human body. Next to qualitative properties like compatibility or stability, other material characteristics influence the cellular interactions taking place at the interface. Especially, surface chemistry, elasticity, porosity and topography have a significant effect on the attachment, proliferation and differentiation of different cell types and can control shape, size and density of cells. Therefore, the following section describes and explains the influence of different parameters and provides an overview of the involved processes.

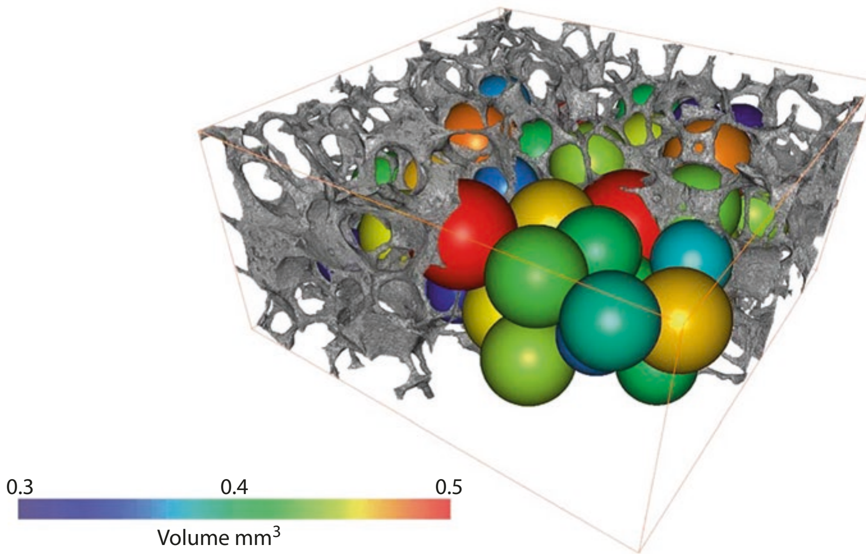


Fig. 6.6 Depiction of the pore volume inside a ceramic-based scaffold obtained using Avizo Fire software [6]

6.3.1 Porosity

Generally, porosity is defined as the void space in a material, whereas for cell culture the accessible porosity of a scaffold is the total amount of void space that culture media or body fluids can enter and pass through it. The pore geometry, size distribution and interconnectivity depend on the nature of the biomaterial and the fabrication process. Besides the pore size, the interconnectivity guides the tissue formation through the scaffold. In that context, the scaffold architecture plays an important role in cell behaviour and tissue ingrowth. One example of a highly porous scaffold with a porosity of 90% is shown in Fig. 6.6. The diameter of the pores was calculated to range between 690 and 1090 mm, whereas the pore volume is seen to vary between 0.175 and 0.675 mm^3.

The methods to characterize porosity and pore sizes are explained in the review of Karageorgou and Kaplan [7]. Adequate measurements and techniques are gravimetry, mercury intrusion porosimetry, liquid displacement method as well as analysis of images from scanning electron microscopy (SEM) or other microscopic methods.

6.3.2 Degradation

Joint replacement materials (e.g. for hip joints) should ideally remain inert for years, whereas for other applications (e.g. osteosynthetic screws), degradability in the body is required. The degradation of biomaterials involves both chemical dissolution (physicochemical degradation) and resorption (cellular degradation by, e.g. osteoclasts, macrophages or fibroblasts). Within the efforts of regenerative medicine towards a *restitutio ad integrum*, biomaterials or even cell-loaded scaffolds that are implanted for the substitution of large defects should be completely degraded within an adequate period of time.

Generally degradation can occur due to several mechanisms: mechanical degradation (e.g. wear) and chemical degradation (enzymes, corrosion). The wear degradation in, e.g. total hip joints (wear of the articulated surface) plays a critical role in the lifetime of an artificial hip joint. Hard-on-hard bearings such as ceramic-on-ceramic (CoC) or metal-on-metal (MoM) are technically promising solutions for this wear problem. This helps to develop new materials with tailored degradation behaviour. As mentioned before, there are different methods to describe the behaviour in vitro (ISO 10993) and in vivo (interface with cells and material).

6.3.2.1 Ceramics

Inert ceramics (e.g. Al_2O_3 or ZrO_2) show no degradation behaviour, whereas some "relatives" of hydroxyapatite (natural compound of the bone) such as tricalcium phosphate (TCP) or other calcium-alkali-orthophosphates are degradable in the body. Factors such as degree of micro- and macroporosity, crystallinity or chemical composition are factors which influence the degradability. One general characteristic of a bone substitute material used for bone regeneration is to be resorbed by osteoclast cells and replaced by new bone. For all bone substitute materials, it is essential that they are included in anabolism or tissue formation and catabolism or remodelling process [8]. In this context materials like calcium carbonate, hydroxyapatite (HA), bioactive glasses (BG), TCP, biphasic ceramics of HA and α- or β-TCP are of interest.

6.3.2.2 Metals

Corrosion is always a concern if using metallic implants. In most of the cases, the current research aims to minimize corrosion and effects of degradation because the degradation products (wear debris, colloidal complexes, free metallic ions, inorganic metal salts, oxides and hydroxides) might elicit toxic or hypersensitivity responses in the body. The effect of metal particles on bone cells and bone resorption is a crucial issue for orthopaedic implant durability, and some patients with metal implants show an elevated metal body content found in the body fluids and remote organs [9].

6.3.2.3 Polymers

Degradable polymers (e.g. polyglycolide acid [poly(L-lactide, poly(D-lactide PLGA, poly(c-caprolactone as well as collagen, gelatine or alginate) typically contain linkages susceptible to hydrolysis. If these materials are also hydrophilic and absorb water, they will degrade. Body enzymes can support these reactions. The degradation products such as gases and salts have to be non-toxic as well. This degradation effect can be used for drug delivery (where drugs are encapsulated into degradable polymers), surgical suture materials (the polymer degrades slowly with a rate which matches the rate of tissue regeneration) or as scaffolds in tissue engineering (the polymer degrades in the body once it has served its purpose of being a template for the regeneration tissue).

6.3.2.4 Composites

Composites are combinations of different material groups. Their degradation behaviour is connected to the degradation behaviour of their individual components. In order for a biomaterial to be successfully used, the degradation products and release rates have to be well characterized and controlled. The degradation mechanisms of each phase need to be understood to design a biodegradable composite for intended use as a temporary implant material.

6.3.3 Wettability

As cellular interactions take place between adhesive molecules and cells, the contact between cell and material can be described as an indirect reaction and is mediated by the protein adsorption on the surface of the biomaterial. Protein adsorption is a very rapid process driven by van der Waals forces, hydrogen bonding and electrostatic and hydrophobic interactions [10] and takes place within the first seconds of contact with biological fluids (like blood or culture medium). Proteins, which show the highest mobility (e.g. albumin), first attach to the surface and are replaced after time by proteins with higher affinity (e.g. fibrinogen) [11], described as the Vroman effect [12]. Due to the high sensitivity of the cell-material interaction, cells can attach and proliferate, or inflammation can cause cell death and rejection reaction in the worst case, depending on the adsorbed proteins on the surface [13]. One of the most important parameters influencing the behaviour of protein adsorption is hydrophilicity and accordingly hydrophobicity of a material, describing the wettability of a surface. The wettability of a material can be determined by static or dynamic contact angle measurement. Thereby, a sessile water droplet with defined volume is deposited on a flat surface. By establishing a tangent line between surface and droplet (Fig. 6.7), a contact angle θ can be determined. Small contact angles ($\leq 90°$) correspond to surfaces with hydrophilic behaviour, whereas high contact angles ($\geq 90°$) describe surfaces with hydrophobic character [14].

In general, proteins have a higher affinity to adsorb more strongly to hydrophobic than to hydrophilic surfaces [15]. Adsorption is a spontaneous reaction and can be described by the following equation:

$$\Delta_{ads}G = \Delta_{ads}H - T\Delta_{ads}S < 0$$

where G, H, T and S are the Gibbs free energy, enthalpy, temperature and entropy, respectively. Δ_{ads} describes the net change of the parameters.

This behaviour can be attributed to the structure of proteins. Proteins are macromolecules consisting of different amino acids with varying side chains (hydrophobic and hydrophilic residues). In aqueous media, the hydrophobic parts of the amino acids tend to be located inside the protein core to minimize the conformational entropy, whereas the hydrophilic parts outside interact with water molecules resulting in water solubility of proteins. Due to interactions between sorbent hydrophobic surface and hydrophobic side chains, proteins unfold their amphiphilic structure resulting in an increase of the entropy which is also the driving force for the protein adsorption. The hydrophobic part of the

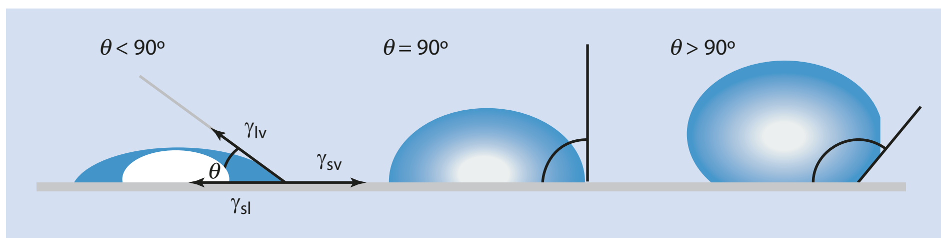


Fig. 6.7 Illustration of the measurement of the contact angle by creating a tangent line at the liquid-vapour interface, γlv, γsl and γsv describe the liquid-vapour, solid-liquid and solid-vapour interfacial tensions, respectively [14]. Reprinted with permission of Springer Link, tbc

protein core covers the hydrophobic surface to reduce the hydrophobic surface area exposed to the solvent [15] attributed to thermodynamic forces. Shortly summarized, interactions of proteins with hydrophobic surfaces are an energetically more favourable reaction compared to hydrophilic surfaces because of the gain of entropy.

6.3.4 Stiffness

Mechanical properties are an important part of tissue engineering to characterize different materials. They give information about the quality of a sample, how easy it is to handle or whether it is load bearing or not. These properties adjudicate for which application and in which environment the material is adaptable as well as if cells are able to grow on it or drugs can be incorporated and subsequently released [16]. Stiffness, also termed rigidity, elasticity or pliability, is a determining factor for cell fate especially in a 3D environment where the cells are surrounded by matrices. The substrate stiffness is sensed by cells through bidirectional ways: cells exerting stresses on the surrounding matrix during tissue regeneration, morphogenesis and differentiation and natural function through the ligand-integrin interactions between cells and the surrounding matrix. Simultaneously, the surrounding matrix exerts stress on the embedded cells, which depends not only on the cell-matrix bonds but also on the stiffness and density of the matrix. The stiffness of the surrounding matrix and the cell-matrix interactions through the ligand-integrin bonds, dictate the extent to which the embedded cells can contract the matrix [17, 18]. The degree of contraction of the surrounding matrix by the embedded cells is one of the determining factors of cell migration, proliferation and spreading in 3D matrices. Cells encounter less resistance in softer and compliant matrices than in stiffer matrices, which has been demonstrated by Bott et al. [19], showing that fibroblasts exhibit higher spreading and proliferation within softer than comparatively stiffer PEG hydrogels. The stiffness of soft biomaterials can be measured using different methods like atomic force microscopy (AFM) or nanoindentation, which will be described more in detail: The technique normally uses a probe, which is brought in contact with the material surface as a first step. There are a range of parameters that can be varied in this technique, i.e. different methods, tip diameters, local differences in the samples because of their heterogeneity, storage media used, temperature, hydration state, time between measurement and tissue excision/death, etc.; hence different results can be achieved. In the next step, the probe is pushed into the material and retracted. During the whole process, the load, displacement and the time are recorded (Fig. 6.8). The parameters are analysed with a range of models such as elastic or viscoelastic. The elastic model is used for materials that return to their original shape when the loads are removed and the unloading path is the same as the loading path, except for viscoelastic materials, which deform after applying and removing a load.

6.3.5 Roughness

It is well known that the micro- and nanotopography of a substratum controls cell behaviour [20]. Convex surfaces can be covered by cells (Fig. 6.9a), whereas cells tend to bridge concave structures (Fig. 6.9b). This was described by 3D printed HA granules for tissue engineering [21]. Furthermore, different nanostructures like gratings, pits or pillars

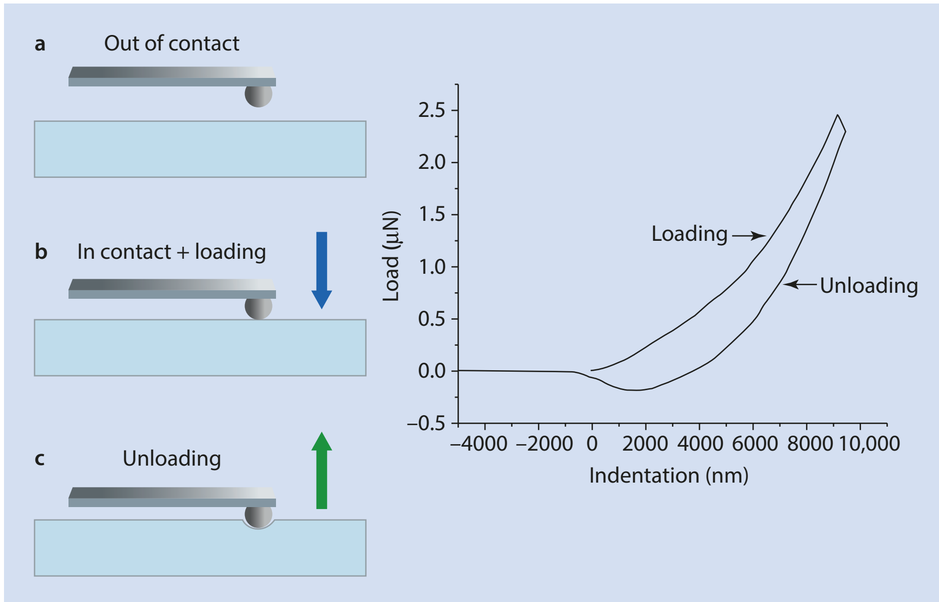


Fig. 6.8 Illustration of the three steps during one indentation cycle (*left* image): **a** approach, **b** loading and **c** unloading (Source: Optics 11, Amsterdam). Typical load – displacement curve for a nanoindenter measurement (*right*)

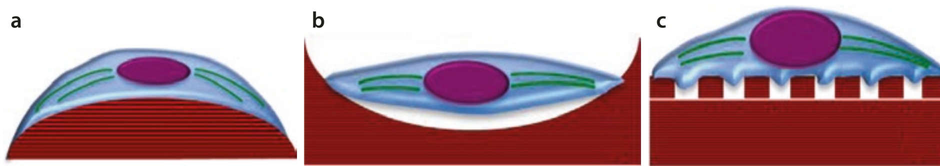
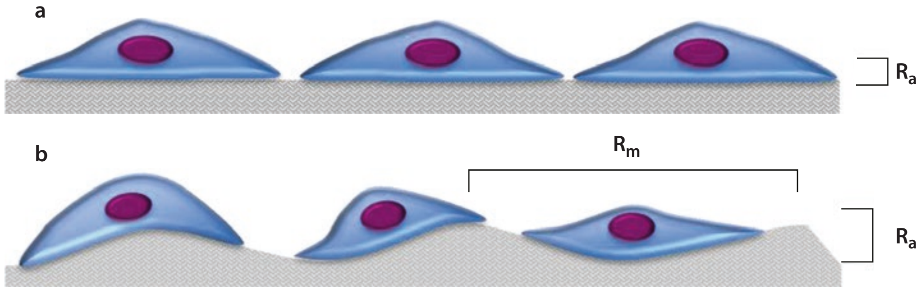


Fig. 6.9 Convex **a** and concave topographies **b** as well as nanostructures **c** can guide cell attachment and cell development. (Images drawn by Dr. Aldo Leal-Egaña, Institute of Biomaterials, Friedrich-Alexander-University Erlangen-Nuremberg)

could guide cell adhesion (75–120 nm) or osteogenesis (300 nm), whereas small structures below 35 nm seem to inhibit cell adhesion [22–24].

The microscopic topography of a biomaterial surface is typically characterized by a series of peaks and valleys, which can be quantified using or 2D profile. Roughness average (Ra, defined as the integral of the absolute height values of peaks and valleys along the evaluated profile), vertical parameter (Rz, mean height from peak to valley along the roughness profile) and horizontal parameter (Rm, average interpeak distance along the roughness profile) are used to describe a surface profile (see also Fig. 6.10). For example, cell proliferation and osteogenic differentiation of myoblasts and osteogenic cells will be affected by the applied surface structure [25]. As compared with micron-scale surface structure, submicron-scale surface structure enhanced osteogenic differentiation of osteoblast-like cells, while it enhanced cell proliferation of osteoblasts [26]. In general, it seems that an optimal spectrum of Ra roughness for bone-forming cells ranges between 0.2 and 2 µm [20]. On the other hand, the surface roughness needs to be optimized for each case of positive cell surface interaction.



■ **Fig. 6.10** Schematic drawing of the influence of different rough surfaces on cell attachment. **a** On smooth surfaces cells will adhere fast and spread with flat morphology. **b** On a rougher surface, cells have to spend more energy to cover the surface. (Images drawn by Dr. Aldo Leal-Egaña, Institute of Biomaterials, Friedrich-Alexander-University Erlangen-Nuremberg)

In summary, specifically tailored biomaterials with organ-specific functions, like porosity, degradation behaviour, wettability, stiffness, roughness and geometry, should be available not only to provide structural support to targeted cells but also to instruct or guide cells in their expansion and tissue formation function.

6.4 Tissue Engineering and Biofabrication

The term tissue engineering (TE) is mentioned in many chapters of this book. TE can be defined as, using the definition of Langer and Vacanti, "an interdisciplinary field that applies the principles of engineering and life sciences toward the development of biological substitutes that restore, maintain, or improve tissue function or a whole organ" [27]. Macro-, micro- and nonporous biomaterial scaffolds have gained much attention since they provide cells not only with mechanical support, growth factors and nutrients but also with a microenvironment for cell homing and for performing biological functions. Particularly, biodegradable polymers, bioceramics and bioactive glasses as well as composites have been widely used as scaffold materials since they could serve their functions only for a defined period. After scaffold fabrication and sterilization, cells with or without growth factors will be seeded onto the scaffolds. In some cases, the cells, scaffolds and growth factors are all mixed together at once, allowing the tissue formation. The basic principles of TE are illustrated in ■ Fig. 6.11.

In general, the cells of a donor organ are seeded and the scaffold is used to guide the new tissue formation. This process has been used to bioengineer heart, liver, bone cartilage, lung and kidney tissue [28]. This regeneration approach seems to be promising combining a patient's own cells to make customized organs that will not be rejected by the immune system.

The main goal in biofabrication approach is to build living tissue substitutes on demand. In order to create functional tissue structures, additive manufacturing technologies are being increasingly considered. They allow the generation of functional structures created out of CAD models within a short period of time and with a very high precision.

The basic concept of biofabrication is the combination of a matrix (hydrogel) with living cells or bioactive molecules and additive manufacturing (3D printing techniques) in order to generate cells in a three-dimensional environment (■ Fig. 6.12). The fabricated products

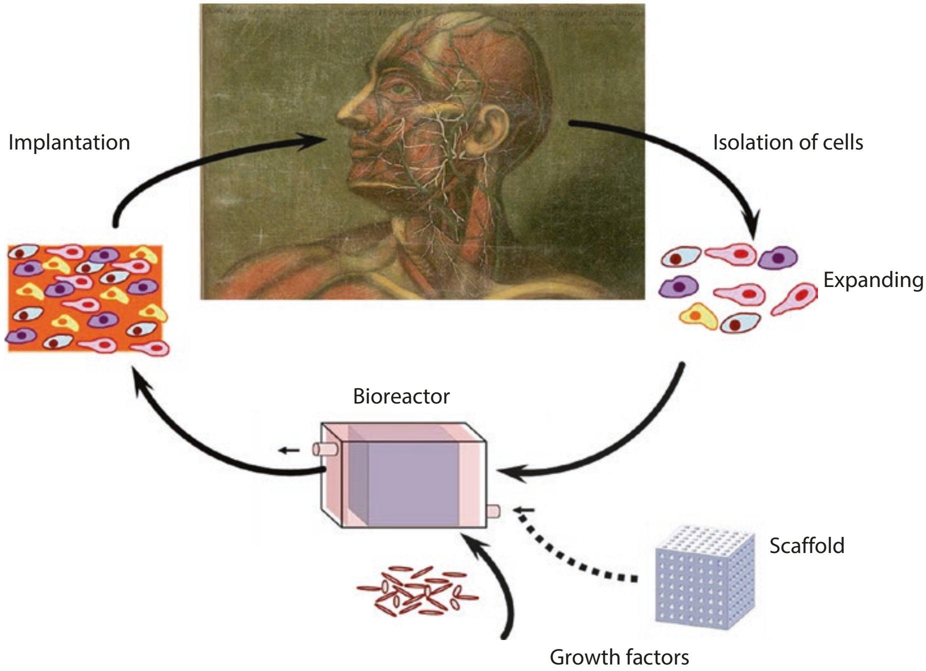


Fig. 6.11 Scheme of the tissue engineering dogma: After their isolation and expansion, cells are seeded on a suitable scaffold. Growth factors and bioreactors can stimulate the attached cells to differentiate into the target tissue type before the construct will be implanted

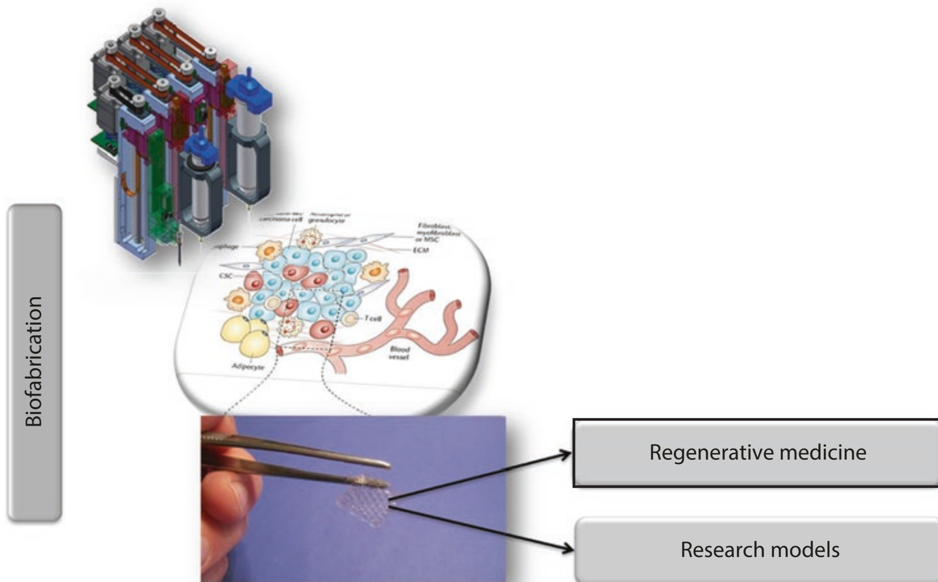


Fig. 6.12 For biofabrication, hydrogel is mixed with cells, and afterwards this solution will be processed by additive manufacturing to form a three-dimensional cell-containing construct for the use in regenerative medicine and for research models

can be used for tissue engineering approaches, like regeneration of tissue or model systems for drug and disease research. The scaffold acts as a supporting structure with an interconnected pore network for the cells. This means that the cells are effectively organized and attached in a three-dimensional matrix. Furthermore the scaffold should support the migration, growth and differentiation of cells. Therefore, it is crucial to consider the physicochemical properties, morphology and degradation behaviour. Different techniques are already established to build three-dimensional (3D) complex cell-loaded structures [29].

One promising biofabrication approach is the use of a hydrogel system based on oxidized alginate covalently cross-linked with gelatin (ADA-GEL). This system is utilized to design biodegradable tissue engineering scaffolds, in which enhanced cell growth, proliferation and migration was observed [30]. Moreover, the plotted ADA-GEL hydrogel constructs exhibit a hierarchy of pore sizes from the nano- to the micrometre range. The biological properties of these hydrogels were studied by comparing the viability and morphology of MG-63 osteosarcoma cells, encapsulated in gelatin and RGD-modified alginate, as reported elsewhere [31].

In conclusion, the key parameter for the success of the bioprinting approach is the biocompatibility of the substrate. The development of cells on a biomaterial surface can be tailored by surface properties. Tissue engineering and biofabrication applications will open more complex but also more efficient insights into the *cell meets surface* issue.

Take-Home Messages

- Biocompatibility is a complex material property, which has been determined by in vitro, in vivo and clinical tests.
- Biomaterials are manufactured by synthetic or biological-derived materials.
- The sterilization of biomaterials is essential, and the selection of the appropriate sterilization method is determined by the used material composition.
- The adjustment of porosity, degradation, wettability, stiffness and roughness are applied to tailor cell-material interaction.
- Biofabrication generates living tissue and includes the perfect interplay of bioink (hydrogel), cells and additive manufacturing.

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Cell–Surface Interactions

Megan Livingston and F. Kurtis Kasper

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What You Will Learn in This Chapter

The interactions that can occur between a cell and an adjacent biomaterial in culture are vast and depend on the biochemical, physical, and mechanical properties of the biomaterial. The present chapter will discuss the effects of key biomaterial properties on cell attachment, proliferation, migration, and differentiation in culture. These cell–surface interactions will be discussed in the context of the envisioned clinical application of the biomaterials to facilitate an improved understanding of the "big picture" considerations when studying biomaterial-based technologies in cell culture, with a particular focus on tissue engineering technologies.

7.1 Overview

When studying cell–surface interactions, primarily in the context of cell interactions with biomaterials in culture, it is important to consider the clinical significance of the technology you are studying/developing. The biocompatibility of a biomaterial generally will be assessed on the pathway to development of a product or technology for a clinical application, especially if the envisioned application involves some interaction with living tissue in a specific context. Biocompatibility is not an inherent material property, but it depends on the intended biological environment and the interactions that occur between the material and its surrounding environment in vivo [4].

Within a biological environment, the chemical and physical properties of material surfaces exert a considerable influence on cell adhesion, proliferation, migration, and differentiation [124]. Cells in a complex organism are surrounded by a multifactorial extracellular architecture of proteins, polysaccharides, and proteoglycans that constantly undergo change due to assembly, remodeling, and degradation events. The intrinsic flexibility of this structure allows the microenvironment to be modified locally in response to unique patterns of protein secretion and modification. Cell adhesion to specific components of the extracellular matrix (ECM) via integrins, cadherins, and discoidin domain receptors activates signaling programs specific to the conformation and composition of that ECM [70]. Cell shape and ECM stiffness govern membrane properties like curvature and tension [90, 93, 99]. These characteristics, in turn, determine membrane composition, protein distribution, and intracellular trafficking [28, 99].

In an in vivo environment, the distribution of signaling molecules and interstitial pressure facilitate formation of molecular gradients that can variably influence cells within a tissue matrix based on their location. These gradients are established through cell secretion, protein diffusion, and proteoglycan-mediated stabilization [3, 13, 62, 93]. Additionally, topographical cues presented by the ECM directly affect cell behaviors such as adhesion, migration, cytoskeletal arrangement, and differentiation [40]. Cells are inherently sensitive to local microscale and nanoscale topographic and molecular patterns in the ECM, a phenomenon called "contact guidance" [47]. Cells respond to physical cues from their surrounding environment manifested through ECM characteristics including adhesive ligand density and pattern, rigidity, and dimensionality and anisotropy [21].

In an in vitro culture setting, an artificial microenvironment for cells is formed by a complex combination of factors, including cytokines, scaffold material, cell–cell interactions, and physical stress [38]. Proteins and biomolecules are key factors that determine

cell–material interactions, and these will nonspecifically adsorb to material surfaces from culture medium. Nonspecific interactions between a cell and a surface do not require a receptor, i.e., only physical attachment. Electrostatic, van der Waals, and hydrophobic forces mediate nonspecific cell–surface adhesion. Proteins typically adsorb to the surface of a biomaterial in a nonspecific way [91, 124]. Therefore, unless the surface of a biomaterial has been chemically modified with ligands/peptides for attachment to specific cell membrane receptors, interactions between cells and a material in culture will be nonspecific.

7.1.1 Stem Cells in Culture for Tissue Engineering

One of the most common clinically driven applications requiring study of the interactions of biomaterials and cells in culture is tissue engineering. With that in mind, this chapter will emphasize technologies utilizing a biomaterial scaffold to encourage regeneration or repair of an individual type of tissue or an entire organ structure. In this realm of research, scientists commonly study the interactions of materials and stem cells (i.e., cells capable of self-renewal and differentiation into specific cell types) and/or progenitor cells in an effort to understand how such cells are affected by cues from their surrounding environment. Unfortunately, the quantity of environmental cues and mechanisms possibly directing stem cell fate has made these cells difficult to control in vitro and in vivo [71].

In tissue engineering, many types of cells have been studied in an attempt to decipher/control stem cells on biomaterial surfaces. Hematopoietic stem cells (HSCs) give rise to all blood cell types [122], while human bone marrow-derived mesenchymal stem cells (MSCs) can differentiate into osteoblasts, chondrocytes, and adipocytes. In vitro, MSCs can be differentiated into various cell types including cardiomyocytes, neuronal cells, and others, which they generally are not capable of becoming in vivo; this phenomenon is called "trans-differentiation" or "stem cell plasticity" [72]. More recently, induced pluripotent stem cells (iPSCs) have been shown to differentiate into various cell types associated with the three germ layers, supporting the immense potential of iPSCs with regard to applications in regenerative medicine [120]. Producing a homogenous resulting cell population when working with stem cells remains a high priority for implantation, drug screening, and/or disease treatment [96].

7.1.2 Scope

This chapter will highlight examples of interactions of cells with naturally and synthetically derived biomaterials, as well as combinations of the two, in the context of tissue engineering. For the purposes of this chapter, naturally derived biomaterials include scaffolds constituted from tissue/organ ECM as well as bulk materials fabricated from individual components of mammalian ECM. Systems based on nonnatural substances, specifically synthetic polymers, are considered to be synthetic biomaterials. While metals and ceramics might additionally qualify as synthetic biomaterials, this chapter will focus on cell–surface interactions with polymers due to the historical emphasis of polymeric materials in tissue engineering applications.

7.2 Naturally Derived Biomaterials

The selection of an appropriate biomaterial is driven by the specific requirements associated with the intended use of the material. Clearly, a biomaterial can present benefits and shortcomings in the context of a given application; it is the responsibility of the researcher to determine if the advantages of a material outweigh its disadvantages through testing and analysis. Given the breadth of clinical needs driving the development of biomaterials, the research community continues to invest tremendous effort in studying the interactions of cells with biomaterials in a wide range of contexts.

Biomaterials can be classified generally into two groups: namely, naturally derived and synthetically derived biomaterials. Many researchers propose that naturally derived biomaterials offer unique advantages for select tissue engineering applications because they can present natural biological features that synthetic biomaterials generally lack. Scaffolds comprising naturally derived materials have been widely studied in regenerative medicine and tissue engineering because it has been suggested that there is no better way to replace a tissue than with its homologous structure [69]. This school of thought lends itself to the notion that tissue engineering scaffolds ideally should mimic the native ECM and actively interact with cells to direct cell adhesion, proliferation, migration, and differentiation toward a desired outcome [73].

7.2.1 Decellularized Organs/Tissues

Some investigators seek to leverage the biological cues naturally resident in the extracellular matrix of organs and tissues by decellularizing the structures and applying the resulting biomaterial construct as a scaffold to direct cell behavior in an organ- or tissue-specific manner. For example, "whole-organ engineering" involves the removal of cellular components (decellularization) from an organ followed by repopulation of the resulting scaffold with specific cell populations. This approach confers the potential to increase the number of available organs for therapy or transplantation, assuming the functionality of the recellularized organ or tissue can be restored [5, 6, 25, 101, 118]. The concept has been applied to decellularize organs and tissues toward the production of scaffolds to support the repair or regeneration of a variety of tissues and organs.

These scaffolds represent the secreted products of cells in each tissue or organ, transducing functional signals deployed through integrins and other cell surface receptors [11, 26, 42, 79]. Biologic decellularized scaffolds (see Fig. 7.1 for an example) retain a

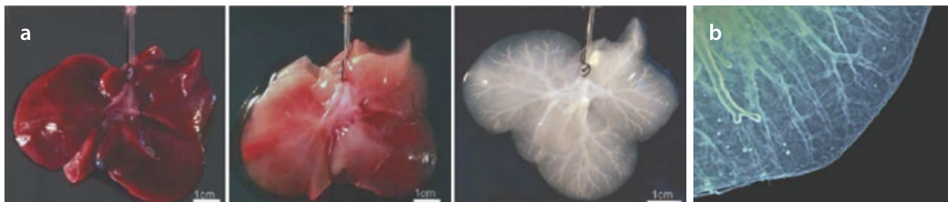

Fig. 7.1 Preparation and ultrastructural analysis of an example acellular liver bioscaffold. **a** Macroscopic view of a decellularized animal liver before, during, and after the decellularization process. Scale bar = 1 cm. **b** View of a decellularized lobe of the liver exhibiting clear parenchyma, defined liver capsule, and vasculature. (Adapted from Baptista et al. [8] with permission from Wiley)

plethora of bioactive molecules and can facilitate tissue reconstruction by encouraging cellular attachment, proliferation, and differentiation via the ECM molecules and proteins retained in the scaffold from the original tissue. Decellularized scaffolds can attenuate intracellular signaling pathways and cell transcriptional events, imbuing cells seeded in these scaffolds with important characteristics of tissue identity [112]. Although the cellular and molecular mechanisms by which decellularized scaffolds facilitate tissue reconstruction are only partially understood, a body of literature has suggested that an ability to recruit stem/progenitor cells may contribute to an improved remodeling outcome [112].

Early work has generated considerable excitement based on assumptions that both cell lineage and fate could be directed by decellularized scaffolds and that functionally appropriate cellular connections could be made [112]. Important examples of decellularized scaffolds are whole or partial lungs, small intestinal submucosa, acellular dermis, bladder acellular matrix, and amniotic membrane. These types of scaffolds have been shown to quickly incorporate with the surrounding tissue, induce the recruitment of cells and deposition of additional ECM, and accelerate angiogenesis, a critical factor for tissue survival in vivo [4, 63].

7.2.1.1 Decellularization Methods

Common methods for the production of decellularized scaffolds include [112]:

- *Physical method*: This approach typically involves repeated freeze–thaw cycles in order to rupture cell membranes and induce cell lysis. It could also include preparation of frozen or freeze-dried material and/or treatment with hypertonic saline or irradiation. Although freeze–thaw techniques can kill cells, dead cells are not necessarily removed by these approaches, and, therefore, they may require augmentation using other decellularization procedures [112].
- *Chemical decontamination method*: The common agents for chemical decontamination are classified generally as either nonionic or ionic detergents. Nonionic detergents (e.g., Triton X-100) may disrupt lipid–lipid and lipid–protein interactions, but largely leave protein–protein interactions intact, so a tissue or organ treated with a nonionic detergent should retain functional protein conformations [97]. Ionic detergents (e.g., sodium dodecyl sulfate, SDS) are commonly used as a supplement to nonionic detergents. Ionic detergents have been known to destroy cells by dissolving in the cytoplasm and breaking up the nucleus and membrane by disrupting protein–protein links, denaturing the proteins [97]. Compared with other detergents, SDS yields a more complete removal of nuclear remnants and cytoplasmic proteins, while disrupting native tissue structure in the process [112].
- *Enzyme digestion method*: Examples include treatment with trypsin, pepsin, collagenase, DNase, and RNase [78].

The freeze–thaw method combined with the chemical method is considered to be one of the most effective approaches to generate decellularized allografts of more fragile tissue, at present, as this procedure effectively removes cells while retaining a complete ECM [112]. Some researchers have suggested that optimal decellularization protocols require a combination of physical, enzymatic, and chemical treatments to eliminate as much of the cellular residues as possible to make the construct safe for transplantation [69].

Unfortunately, evidence advises that the vast majority of current decellularization techniques present some limitations in terms of weakening the decellularized matrix [69]. Many of the utilized detergents affect crucial proteins present in the ECM. Non-denaturing,

nonionic detergents have the least damaging effect on collagens, elastins, and laminins, whereas denaturing and zwitterionic detergents deplete these critical structural ECM/basement membrane proteins in both the vascular and airway compartments, hindering the ability of a scaffold to revascularize [102]. Overall, decellularization protocols still need to be refined and standardized to better maintain the integrity of tissue vasculature, in particular that of delicate capillary beds, to decrease the potential for complications, including hemorrhage, upon implantation [102].

7.2.1.2 Shortcomings of Decellularized Matrices

Decellularization of tissue/organs often damages the ECM to some degree [102]. For example, lungs perfused with 0.1% SDS during decellularization suffered a loss of ~80% of collagen and 90–95% of glycosaminoglycans [86, 87]. Damage to these critical ECM components decreases reseeding potential by presenting fewer cell attachment proteins, impeding the potential of the scaffold for success in vivo. Additionally, recellularization of complex tissue (e.g., whole organs) is extremely difficult because the recellularization process is typically dependent on perfusion, which impedes precise control of cell attachment, movement, and proliferation [51].

Decellularized matrices can be very intricate structures, making it quite difficult to characterize which specific ECM components are driving the desired effects. In an effort to simplify the system in vitro, some researchers carry out less complex approaches to leverage naturally derived materials to present certain elements of the ECM environment to controllably and reproducibly drive cell behavior.

7.2.2 Extracellular Matrix Derivatives: Natural Polymers and Hydrogels

Cells attach to the ECM through integrin receptors, and engagement of these receptors initiates multiple intracellular signaling cascades that regulate cell survival, proliferation, and differentiation [4, 32, 43]. It is often advantageous for biomaterial scaffolds to mimic particular characteristics of the natural ECM [73]; therefore, some researchers employ materials composed of bulk ECM derivatives in order to direct cell actions on material surfaces as an alternative to using the whole ECM presented by decellularized organs and tissues.

Natural polymers derived from extracellular matrix components (e.g., collagen, gelatin, fibrin, and hyaluronate) contain specific molecular domains that can support and guide cells at various stages of their development, possibly allowing enhanced biological interactions of the scaffold with cells and the host environment [80]. To study cell adhesion in 3D environments, many techniques have been developed to encapsulate cells in hydrogels (hydrated polymer networks that behave as viscoelastic solids) [93]. Cells in 3D respond differently to exogenous growth factors than they do in 2D [93, 111, 121], enabling the hydrogel structure to be morphologically similar to that of the ECM when used to encapsulate cells [12]. Natural hydrogels are therefore used in a variety of tissue engineering applications to direct the behavior of encapsulated cells. The interested reader is directed to ▸ Chap. 6 in this textbook for additional discussion of differences between 2D and 3D culture.

In addition to biochemical cues, the mechanical cues presented by naturally derived material can impact interactions with cells. For example, the mechanical properties of

natural polymers and hydrogels can be modified via cross-linking to match the design criteria necessary for a variety of tissue regeneration applications. Generally, the stiffness of the material increases with the degree of cross-linking in the material, which can in turn impact cell phenotype. Accordingly, one must balance consideration of the potential effects of biochemical and mechanical cues presented by a material to cells within the context of the envisioned application.

Collagen-based hydrogels, for example, are considered to be a favorable biomaterial for both cartilage and bone scaffolds, as collagen is the predominant protein in the ECM of these tissues [4]. Cell attachment to collagen can be altered by chemical modification, including the incorporation of fibronectin, chondroitin sulfate, or low levels of hyaluronic acid into the collagen matrix. Using these components to formulate collagen hydrogels has resulted in biomaterials designed for reconstruction of the liver, skin, blood vessels, and the small intestine [4, 65].

7.2.3 Challenges of Naturally Derived Biomaterials

In addition to the challenges with decellularization processes described above, naturally derived materials face many challenges that must be addressed to ensure the widespread clinical utility and success of these technologies.

Decellularized scaffolds must be sterilized before implantation or reseeding in order to eliminate endotoxins and bacteria, virus, or prion presence remaining in the scaffold [92], while preserving the structural, biochemical, and mechanical properties of the ECM [69]. These scaffolds may be sterilized by simple treatments such as incubation in acids [48] or solvents [44], but these methods may not provide sufficient penetration or may damage key ECM components [45]. Other sterilization techniques such as ethylene oxide (ETO) exposure, gamma irradiation, and electron beam irradiation are known to alter ECM structure and mechanical properties [39, 105], including properties of clinical products composed of ECM [46, 77]. ETO treatment can result in the inhibition, inactivation, or destruction of nucleic acids and proteins. As a result, ETO has been shown to decrease the stiffness and increase the maximum elongation of the sterilized scaffold [39]. ECM degradation during irradiation is at least partially attributed to the denaturation of key structural proteins including collagen; this process cannot be mitigated by exposure and even occurs at relatively low doses [105]. This degradation occurs because gamma irradiation additionally causes residual lipids to become cytotoxic [77] and accelerates enzymatic degradation of the ECM [25, 46]. Denaturation of structural proteins arising from sterilization may affect the interactions of the material with cells. These consequences of sterilization techniques should be considered for all biomaterials including a naturally derived component as they may impact the interactions of these materials with cells.

Furthermore, signaling molecules remaining in decellularized tissue are sometimes not sufficient in type and/or quantity to repair large defects, requiring the incorporation of additional signaling molecules to augment those present in the scaffold [73]. In addition, mature tissue matrices often do not present pore structure flexibility to tune the material for quick and uniform cell migration and proliferation throughout, which is generally thought to be essential for successful tissue engineering/repair in many applications [73]. Therefore, decellularized ECM or its derivatives may not be the ideal scaffold for certain tissue engineering applications, as tissue engineering should ideally promote tissue regeneration compared to natural functional wound healing.

Naturally derived materials present unique advantages by expressing biomimetic cues to drive cell interactions, but challenges to their clinical application remain, such as controlling the specific cues present and/or material properties. On the other hand, synthetic materials generally feature tunable material properties but typically lack inherent biologically active domains to drive cell interactions/function. However, various techniques can be employed to integrate biologically active domains or topographic features on synthetic biomaterials in a controlled fashion to direct cell interactions in a tunable way.

7.3 Synthetic Biomaterials

7.3.1 Advantages

Synthetic biomaterials allow material scientists to mimic specific advantageous features of natural ECM by imbuing biomaterials with patterns of adhesion, composition, growth factor and mechanical gradients, cell positioning, degradation rates, and geometry to direct tissue morphogenesis [23, 71, 73]. The intrinsic properties of raw synthetic materials play a strategic role in the production, structure, morphology, and, consequently, the functional performance of the synthetic polymer scaffold [4, 20]. These biomaterials exhibit tunable physical, chemical, and biological properties in order to instruct precise cell signaling, allowing researchers to uncouple these signals in an effort to reduce the complexity of a cell culture system [71]. In other words, synthetic biomaterials allow researchers flexibility to engineer scaffolds with tunable properties to determine the role of specific physiological elements, whether they be physical or chemical.

7.3.2 Disadvantages

Certain disadvantages to synthetic biomaterials must be understood when studying interactions between these materials and cells in culture. Current standard stem cell culture protocols for 2D culture on traditional polystyrene (i.e., tissue culture plastic) do not provide specific interactions, instead relying on nonspecific interactions, which do not effectively mimic the natural microenvironment [113]. Cells normally exist in complex tissues and organ systems fed by blood vessels and substantially affected by environmental changes, such as the expansion and contraction of lung tissues during inspiration and expiration. These conditions cannot be replicated suitably in an ordinary Petri dish [124]. While surfaces can be designed to present epitopes (e.g., cell adhesion peptides) for binding of specific cell surface receptors, it is not clear how long the cell has access to these epitopes in vitro or in vivo [58]. The combinatorial complexity of incorporating various ECM proteins, signaling molecules, mechanical stimuli, and heterogeneous cell populations in different spatial and temporal patterns creates an enormously large search space from which to evaluate the effective engineering of synthetic biomaterials [71].

The following considerations have been attempted to facilitate the overall success of synthetic biomaterials for tissue engineering applications: synthesis of material compositions or properties similar to those of the ECM (i.e., chemical modifications), novel processing techniques to achieve structures mimicking the ECM (i.e., physical modifications), and approaches to emulate cell–environment interactions (i.e., mechanical modifications) [73].

7.3.3 Modification of Synthetic Biomaterials

In general, surface and bulk modifications of materials can be employed to achieve desired cellular interactions with a synthetic biomaterial [73]. Many researchers suggest that understanding and selectively presenting components and characteristics of the in vivo ECM in a cell culture setting can potentially improve the success of biomaterial technologies and help answer fundamental scientific questions [58].

7.3.3.1 Synthetic Biomaterials for Tissue Engineering

When tailoring synthetic biomaterials for specific clinical applications, standards necessary for success in that area must be paramount. For example, synthetic biomaterials for tissue engineering should generally meet the following criteria: (I) three-dimensional architecture, (II) interconnected pores to ensure cell growth and transport of nutrients and metabolic waste, (III) suitable surface chemistry, (IV) controllable degradation and resorption, and (V) suitable mechanical properties [4, 82]. A tissue engineering scaffold should have specific biological properties, including biocompatibility, absence of a chronic inflammatory or immune response, a close interaction with cells, and adequate mechanical properties for the intended application [22].

Mimicking cell niches in vitro may include complex surface characteristics such as topography/chemistry or even more complex approaches such as 3D culture, coculture, flowing media, physical stimulation, or a combination of these [113]. Without proper culture systems and protocols, some cell types cannot grow normally outside the body and will gradually lose their characteristics and function [61]. For example, in the absence of precise control over stem cell differentiation, the likely outcome is a heterogeneous cell population or, with non-fully differentiated pluripotent cells (i.e., iPSCs), the potential to form a teratoma or undesired cell types after transplantation [113].

7.3.3.2 Chemical Modifications to Synthetic Biomaterial Surfaces

Inspired by features of the ECM, researchers have developed numerous chemical surfaces capable of interacting with precise components of biological systems such as proteins, nucleic acids, and cells, many of which allow the fine control of spatial patterning, substrate composition, and tunable length of ligand linkers [89, 124]. Material surfaces can be modified with functional groups using methods such as self-assembled monolayer formation, Langmuir–Blodgett deposition, layer-by-layer assembly, and surface-bound peptides [124].

Different surface chemistries and topographies, particularly at the nanoscale, can change the amount and conformation of protein adsorption, which may subsequently lead to modulated outcomes [35, 53, 55]. Researchers should carefully evaluate the conformation of adsorbed proteins and their resulting effects because they can significantly affect interactions with cells. For example, platelet adhesion and activation on material surfaces can lead to blood coagulation and thrombosis, which largely determine the successful application of a biomaterial in artificial organ implantation and blood-contacting medical devices [104].

Techniques like Förster resonance energy transfer (FRET) and fluorescence lifetime imaging microscopy (FLIM) allow researchers to study protein–protein interactions [93] in order to replicate specific cell–environment interactions in vitro. In particular, FRET has been used to study membrane receptor–ligand binding as well as protein conformation [41, 93, 110]. The use of adsorbed proteins to achieve cell–surface adhesion is a relatively simple approach. However, proteins adsorbed to a surface can denature and may

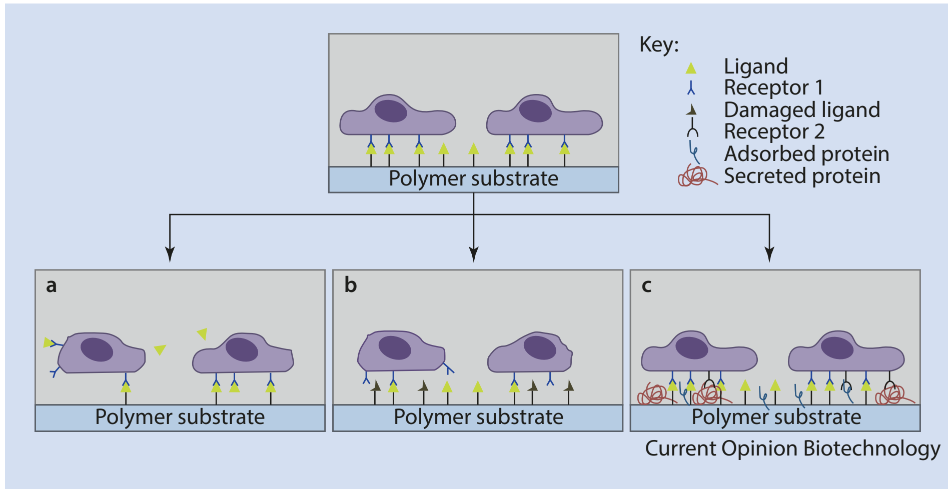

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Fig. 7.2 Changes to surface-attached ligands. Over time, the cell–material interface can be dynamically altered through: **a** removal of cell-adhesive ligands from the surface, **b** degradation/denaturation of the ligand, and **c** unintended or obstructed attachment over time due to secreted or adsorbed molecules. (Reprinted from Krutty et al. [58], with permission from Elsevier)

change in α-helix content, β-sheet content, and structural rigidity [98]. Coatings that form the cell–material interface can be composed of individually purified glycoproteins (e.g., fibronectin) or heterogeneous mixtures of proteins (e.g., Matrigel) [58]. The orientation of these adsorbed proteins is not easy to control [30, 58]. From a shelf-life perspective, the stability and biological activity of protein-coated surfaces can be difficult to retain for long periods of time, undergoing multiple possible changes over time (see Fig. 7.2), the effects of which are furthered during commonly used sterilization techniques such as gamma irradiation [113].

To better control the concentration and population of biologically active sequences on a biomaterial surface, polymer substrates can be modified with a non-fouling layer and short peptide sequences derived from ECM proteins (e.g., collagen, fibronectin, and laminin) or growth factor-mimicking peptides [56, 67, 95]. Common peptide–polymer bonds include amide, carbamate, alkyl sulfide, thioester, triazole, ester, thioether, and disulfide bonds, which may be vulnerable to hydrolysis, displacement, or protease-mediated degradation [17, 58]. Carbamate, thioester, and ester bonds are generally susceptible to hydrolytic degradation at physiological pH, while amide bonds are more stable [58, 94].

Oligopeptides (i.e., short peptides containing up to 20 amino acids) derived from or inspired by ECM proteins have also been widely studied in the context of synthetic biomaterials [108]. Synthesis of oligopeptides is relatively inexpensive, their conjugation onto surfaces is straightforward (e.g., carbodiimide coupling chemistry), and their stability is relatively high (i.e., lower enzymatic degradation than proteins) without having the immunogenicity- and sterilization-related issues of proteins [27]. In addition to native peptides (inspired by or derived from ECM proteins) and semisynthetic peptides (e.g., cyclic RGD), fully synthetic peptide mimetics have recently attracted significant attention due to their consistent properties, lower cost, and high integrin affinity and specificity [84, 85]. Changes to the peptide structure (i.e., linear RGD vs. cyclic RGD) can also increase

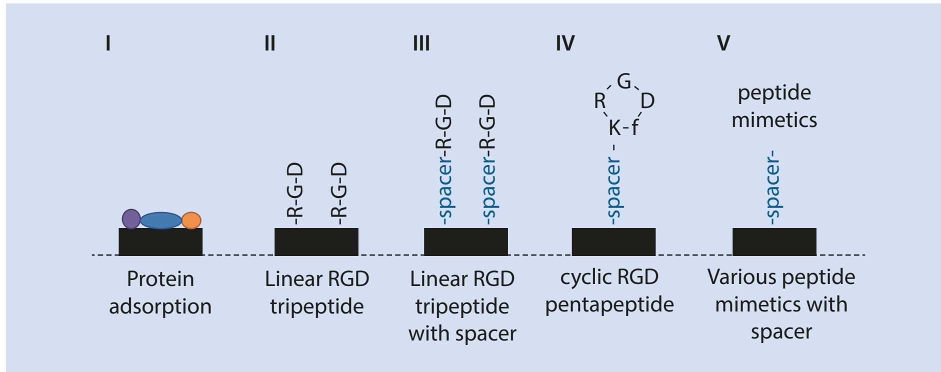

Fig. 7.3 Common examples of surface modification by protein adsorption and peptide grafting. **I** Nonspecific protein adsorption generates a biointerface with multiple signals and binding sites presented in a surface- and time-dependent manner. **II** Linear RGD tripeptide grafted to the biomaterial surface. **III** RGD tripeptide grafted to the surface with spacers to increase the chance for cell recognition. **IV** Grafted semisynthetic cyclic RGD pentapeptide has been reported to have a higher cell affinity than linear RGD. **V** Grafted peptide mimetics with spacers have shown greater cell affinity and are more cost effective and reproducible than native peptides. (Reprinted from Wang et al. [113], with permission from Elsevier)

the specificity of integrin recognition and stimulate other cellular responses such as differentiation and/or phenotype maintenance [57]. A schematic of these types of surface functionalization can be seen in Fig. 7.3.

Patterns and spacing of attached bioactive molecules can also alter cell response. Ligands/peptides spaced too closely together can cause cells to stay relatively spherical while binding sites too far apart may not allow for strong enough attachment or growth of the cells [64]. In addition, the presence of polymer sequence spacers, of varying length and molecular weights, between the material surface and the attached cell has been shown to increase cell attachment and proliferation on synthetic hydrogels [100].

Some synthetic biomaterials lack the reactive groups required for direct covalent functionalization with cell binding sites. These substrates may require additional treatment with plasma, silanization, other chemicals, or coating with a reactive layer [58]. For example, plasma treatment can induce a shift from a hydrophobic to a hydrophilic surface without significantly altering morphology, exposing potential attachment sites for bioactive molecules [73].

7.3.3.3 Physical Modification of Synthetic Biomaterial Surfaces

As chemical modifications to a biomaterial surface can change over time in an uncontrollable and non-reproducible manner, many researchers have studied the effects of physical modifications in order to elicit specific cell functions. For example, the pore size of three-dimensional scaffolds can affect cell binding, migration depth, and phenotypic expression [81]. The porosity of a synthetic biomaterial can be changed to optimize a scaffold for a specific application. However, some polymers undergo bulk erosion, feasibly causing scaffolds to fail prematurely. The resulting burst release of acidic degradation products from some materials can cause a strong inflammatory response [9, 75], warranting consideration of several factors when designing a porous scaffold.

A popular option for porous synthetic biomaterials is nanofibrous scaffolds, whose pores comprise the gaps between nanofibers. Nanofibrous polymer scaffolds advantageously mimic the scale of the natural ECM to enhance tissue regeneration while

circumventing potentially adverse immune responses and pathogens that might be observed with use of natural ECM components [73]. Cells have been shown to adhere to nanofibrous scaffolds at a quantity 70% greater than on solid-walled scaffolds [73, 116]; however cell penetration within nanofiber scaffolds may present challenges.

Nanotopography refers to nanometer-scale physical features of biomaterial surfaces. Because the cytoskeleton connects to the cell nucleus, changes in the cytoskeleton can cause a more direct effect on gene expression and signal transduction than cell signaling via the cytoplasm [113]. It is clear that nanotopographies underneath the attached cells directly interact with their filopodia and lamellipodia due to a similar size scale. Nanogrooves of a similar size to filopodia and lamellipodia can guide cells through receptor–ligand interactions (e.g., integrins) and can trigger complex intercellular pathways (e.g., MAPK-mediated pathways) [10, 54, 76]. In addition, nanoprotrusions are structures frequently found in the native ECM [14]; they can appear as spikes, pillars, and/or hemispheres [113]. The edge of ridges can also accumulate more proteins or alter protein conformation, influencing focal adhesion at the ridge edge and, eventually, cell alignment [10]. Although the native ECM structure is not perfectly ordered and the size is widely distributed, ordered, uniform topographies provide excellent insight into cell–surface interactions in a defined and controllable manner [113].

Nanotopography can induce stem cell differentiation via different intracellular pathways than differentiation media [29]. Therefore, many researchers are using nanotopography as a way to design simple, reproducible synthetic biomaterials for tissue engineering applications. For example, nanotopography alone can induce the differentiation of MSCs into a neuronal lineage and induce a more significant upregulation of neuronal markers compared to microtopography, highlighting the importance of size in topography-induced differentiation [66, 119]. Another research group found that bone morphogenetic protein-2 immobilized on 15-nm-diameter nanotubes promoted osteogenic differentiation, while 100-nm-diameter nanotubes reduced cell adhesion, increased apoptosis, and promoted chondrogenic differentiation [83]. This suggests that stem cells can sense physical material characteristics on the order of tens of nanometers and alter their adhesion profiles [113].

Different methods, including template-based techniques, photolithography, chemical vapor deposition, electrochemical deposition, electron beam lithography, femtosecond laser ablation, and atomic force microscopy tip scratching, are used to fabricate various nanostructures on a wide variety of synthetic materials [113]. However, the pattern area is often very small ($<1\ cm^2$) using these technologies because of the fine features and time-consuming processes involved [113]. Recently, soft lithography has emerged as a popular technique to confer ordered nanotopographies on a biomaterial surface, as it offers control over multiple features in a culture system, including geometry, patterns of ECM protein functionalization, and application of flow [52, 93, 115]. Minor differences in protocols, surface treatments, and cell types can generate different topographic results, and the underlying mechanism of cell–surface interactions is not yet fully understood [113].

7.3.3.4 Modification of the Mechanical Properties of Synthetic Biomaterial

The stiffness of the ECM is known to impact various cell activities from gene transcription and cytoskeleton remodeling to cell–cell interactions [37]. Most cells not only sense but also respond to mechanical properties of the ECM by adjusting their focal adhesion structure, cytoskeleton organization, and overall state [114, 124]. Increased membrane

tension decreases the probability of vesicular budding and favors exocytic merging of vesicles with the plasma membrane [28, 93]. This affects the overall balance of vesicular trafficking within the cell, which can influence diverse signaling pathways such as growth factor receptor signaling, reactive oxygen species production, and phagocytosis [93]. Therefore, evidence suggests, for example, that soft matrices (mimicking the brain) can be neurogenic, stiffer matrices (mimicking muscle) can be myogenic, and rigid matrices (mimicking collagenous bone) can be osteogenic [34].

There are many tools beyond material stiffness that can be used to mimic the in vivo mechanical microenvironment of cells, such as optical tweezers, flow chambers, and micropipettes [124]. A direct method to provide mechanical stimuli to cells is to culture them on elastic membranes, then stretch them, and fix them for immunostaining or biochemical analysis [16]. Additional details regarding dynamic cultivation and mimicking physiology in cell cultures can be found in ▶ Chaps. 8 and 9, respectively.

7.4 Hybrid Materials

As naturally and synthetically derived biomaterials individually suffer from the shortcomings discussed previously, some researchers have developed hybrid biomaterials in an effort to more accurately and reproducibly replicate characteristics of natural tissue, leveraging the advantageous qualities of both natural and synthetic materials. For example, many cell types have been shown to synthesize ECM molecules in vitro, including collagen, elastin, laminin, fibronectin, aggrecan, decorin, glycosaminoglycans, and calcium

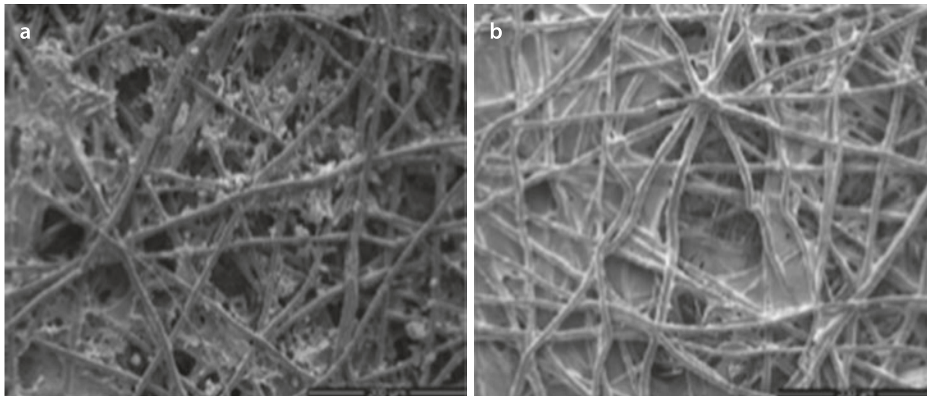

Fig. 7.4 Scanning electron micrographs (250× magnification) of constructs seeded with cells for enough time to establish an ECM, then decellularized via different processing methods to isolate the ECM on a polymer fiber matrix. Constructs presented were processed using the following methods: **a** exposure to ethylene oxide (ETO) for 14 h after 12 days of culture with MSCs, **b** 3 freeze–thaw cycles followed by 14 h of ETO exposure after 12 days of MSC culture, **c** treatment with Triton X-100 and ammonium hydroxide detergents followed by 14 h of ETO exposure after 12 days of MSC culture, **d** 3 freeze–thaw cycles followed by EDTA treatment at 14 h of ETO exposure after 12 days of MSC culture, **e** exposure to ETO for 14 h after 16 days of culture with MSCs, **f** 3 freeze–thaw cycles followed by 14 h of ETO exposure after 16 days of MSC culture, **g** treatment with Triton X-100 and ammonium hydroxide detergents followed by 14 h of ETO exposure after 16 days of MSC culture, **h** 3 freeze–thaw cycles followed by EDTA treatment at 14 h of ETO exposure after 16 days of MSC culture. (Adapted from Thibault et al. [107] with permission from Wiley)

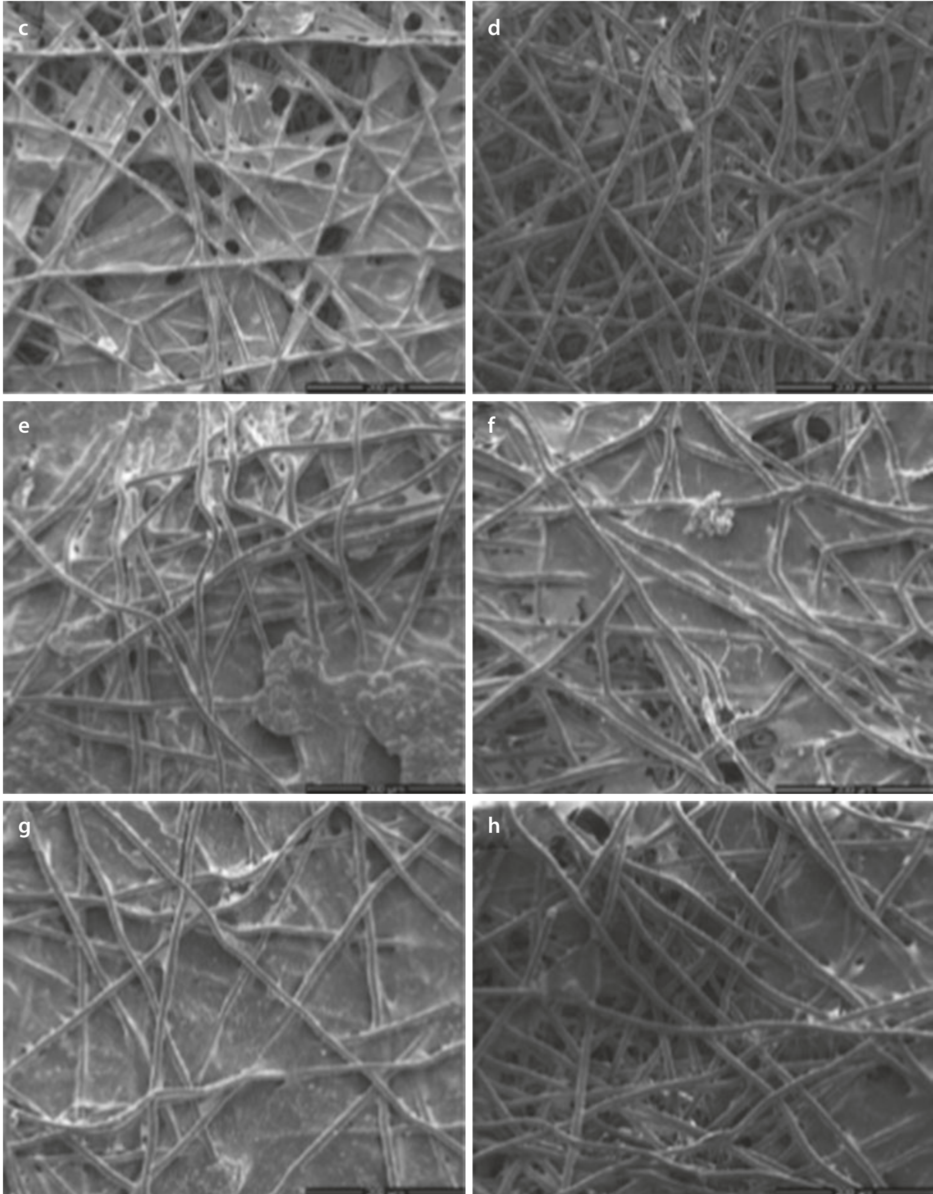

Fig. 7.4 (continued)

deposits [2, 7, 15, 33, 74, 109, 117]. These cell-secreted matrices can then be decellularized and used to direct stem cell differentiation (see Fig. 7.4) [31, 58, 88].

In addition, some researchers have designed natural and synthetic biomaterial composites as biomimetic constructs for various clinical applications, including bone regeneration. Inorganic compounds, such as hydroxyapatite or calcium phosphates, in a synthetic composite scaffold can provide osteoconductivity, while the bulk polymer provides a continuous structure and design flexibility in order to achieve high porosity and surface

area, which are necessary for anchorage-dependent cells to survive and differentiate [68, 73]. While hybrid materials present the potential to advantageously combine the tunability of synthetic materials with the biological activity of natural biomaterials, the complexity of the resulting constructs may present regulatory challenges on the pathway to clinical translation.

7.5 Future Directions

While the plethora of technologies discussed above have allowed for general study of different cell–material interactions, researchers continue to develop novel materials with advantageous characteristics of natural and synthetic biomaterials in addition to abilities not seen with any of the materials previously mentioned.

7.5.1 Bioprinting

Bioprinting combines the biological elements of natural tissue with printing technologies, both 2D and 3D, facilitating the ability of researchers to print various structures. Examples of bioprinted systems being tested include 2D patterned ligand designs for cell attachment in precisely controlled arrangements and 3D hydrogel patches to induce desired stem cell differentiation for various clinical applications. Bioprinting technologies are now capable of printing biomaterials, ligands/molecules, and cells with size resolutions on the micrometer scale and an immensely advantageous ability to tune both the mechanical and physical properties of the printed material (see Fig. 7.5). However, the relative novelty of bioprinting has not allowed sufficient time for consideration and testing, as of yet, of the possible chronic inflammatory and adaptive immunity responses to these types of biomaterials in a range of applications [51].

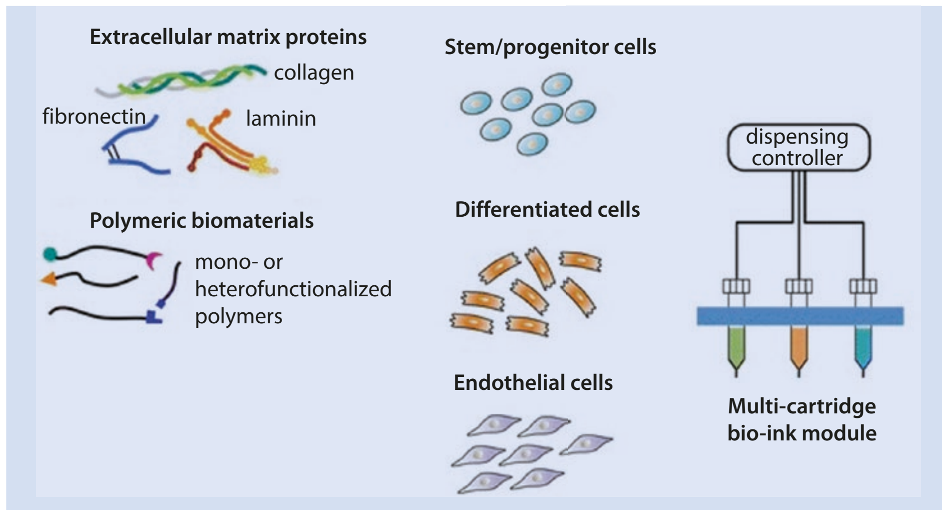

Fig. 7.5 Schematic showing examples of components used as bioinks in 3D bioprinting. This controllable technology aims to manufacture spatially defined tissues or organs across multiple size scales in three dimensions. (Adapted with permission from Jung et al. [51])

7.5.2 Responsive Polymers

Responsive polymers can change their surface physical and chemical properties according to external stimuli, such as temperature change, light irradiation, and electric fields [103, 104]. Examples of types of responsive polymers include thermoresponsive (e.g., 2-diethylaminoethyl acrylate) [36, 123] or photoresponsive (e.g., methacrylated hyaluronic acid) hydrogels [59]. Photoresponsive polymers are photosensitive materials whose physical and chemical properties, such as conformation, shape, surface wettability, membrane potential, membrane permeability, pH, solubility, sol-gel transition temperature, and phase separation temperature, can be reversibly changed by photoirradiation [50, 124].

Poly(N-isopropylacrylamide) (PNIPAAm) [106], a commonly utilized thermoresponsive polymer, can be formulated to support mammalian cell adhesion to the material film and proliferation into a confluent sheet at temperatures higher than its lower critical solution temperature (LCST) of about 32 °C. However, when the temperature of cell culture drops below 32 °C, the cells detach, retaining cell–cell and cell–matrix interactions, and can be harvested as a sheet for tissue engineering applications, something not possible when using trypsin to detach cells in standard culture [1, 18, 19, 60]. Furthermore, the wettability and conformational changes of this polymer could induce corresponding adsorption of ECM proteins above the LCST and desorption below the LCST [24].

7.6 Summary

Many challenges persist in efforts to fully understand cell–material interactions in culture. For one thing, achieving control over the cell–surface interactions and maintaining long-term substrate stability in culture conditions remain difficult [49]. Moreover, there is still only a limited understanding of the dynamics of cell–surface interactions over time [58]. It is generally regarded that the current state-of-the-art culture tools are limited and that this could hinder progress in our attempts to understand how cells function on surfaces and how they can be controlled for clinically relevant applications [113].

New materials, and the properties that their surfaces impart, are anticipated for the next generation of implants, regenerative medicine and tissue engineering devices, as well as biosensors and drug delivery devices for disease diagnosis and treatment [124]. The quantitative characterization of cell-secreted ECM molecules, protein adsorption after surface degradation, and degradation of functional peptides on biomaterials is likely to be a highly significant area of upcoming study. In order to maintain control and biological relevance in vitro, there is a need to create material surfaces designed for real-time characterization of polymer stability, peptide stability, ECM molecule secretion, protein adsorption, and cell-mediated ECM degradation/remodeling. Ultimately, a clearer understanding of cell-biomaterial dynamics may lead to innovative approaches for materials to adapt to cell behavior in a controlled and predictable manner [58].

Take-Home Messages

- Materials exhibit various surface properties that affect how cells react to and interact with the material.
- Biologically active domains present in the ECM of tissues and organs impact cell function and can serve as a guide for developing materials to direct cell behavior.
- Tissues and organs can be decellularized to utilize the secreted ECM proteins and molecules as a scaffold for stem cell programming and differentiation.
- Decellularization procedures can alter scaffold protein content and structure and, in turn, cell interactions.
- Isolated extracellular matrix proteins can be used as bulk materials for scaffolds to direct cell interactions.
- Synthetic polymeric biomaterials feature tunable chemical, biological, and physical properties that can be harnessed to control cell interactions with the material.
- Synthetic materials can be modified with attached peptides and ligands to control cell function.
- Nanotopography on a biomaterial surface can be used to control cell attachment, movement, and stem cell fate.

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Mimicking Physiological Oxygen in Cell Cultures

Nicholas R. Forsyth, Rachel Steeg, Muhammad Ahmad, Mohammed Al Zubaidi, Rakad Al-Jumaily, Marwan Merkhan, and Tina Dale

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What You Will Learn in This Chapter

The *in vitro* mammalian cell culture environment is designed to replicate a wide range of biophysical and biochemical parameters as is sufficient to allow isolation and continued laboratory-based growth. There are a number of parameters to be considered in this regard including, but not limited to, sugar, oxygen, mechanical forces, substrate, nutrients (salts, amino acids, carbohydrates, vitamins, minerals), growth factors, hormones, pH, temperature and osmotic pressure. Some of these components are readily controllable via chemical formulation, while others require greater care to recapitulate and others still are ignored. This chapter will provide an overview of the history of mammalian cell culture followed by a more detailed view of the development and application of tools designed to recapitulate physiological normoxia within the *in vitro* laboratory environment.

The phrase physiological normoxia is used here to describe the normal oxygen values experienced by mammalian cells and tissues *in vivo*. Air oxygen (21% O_2) does not represent a physiologically normoxic value but can instead be considered as being physiologically hyperoxic. The opposite extreme to hyperoxia is hypoxia, and this occurs when oxygen levels drop below that which is ordinarily considered normoxic, i.e. 2% O_2 dropping to $<0.1\%$ O_2. The values presented here are arbitrary, and for helpful perspectives, we recommend the following articles [26, 40].

8.1 A Historical Basis for Modern Cell Culture

Cell culture forms the underlying basis for a great deal of biomedical research over the previous 50–60 years. The roots of mammalian cell culture however can be found over a century ago in an elegant body of work performed by Ross G. Harrison and colleagues [22]. Their breakthrough description, in 1907, noted the successful maintenance of viable frog spinal cord tissue for a period of 4 weeks where lymph clot was used a nutrient source. Their description provided a very early example of *in vitro* tissue culture which can be traced through to the tissue engineering ambitions at play in the twenty-first century. A further 5 years would pass before Alexis Carrel would publish the manuscript which underpins perhaps all of mammalian *in vitro* cell culture [4]. The concept at play in Carrel's work was that supplementation of cells with plasma or serum and strict aseptic technique would enhance *in vitro* growth. Though it is now apparent that additional cells may have been inadvertently added to the culture during supplementation steps, the underlying principles remain sound. The discovery and description of the utility of the serine protease, trypsin, as a convenient enzyme for disaggregating and passaging cells, established baseline principles for *in vitro* culture and expansion of cells [35].

The transition of mammalian cell culture into a whole laboratory endeavour is marked by the 1943 series of publications by Wilton Earle in the *Journal of the National Cancer Institute* describing a complete apparatus including glassware cleaning, glass Carrel flasks for *in vitro* growth, photomicrograph and microcinematography equipment, *in vivo* tumorigenesis and histopathological approaches [7–11, 31]. Though illustrative of approaches to study malignancy, this provided a window into the engineering approaches being utilised and the ingress of technology into the *in vitro* culture environment. This was followed shortly after by the description of methodologies to isolate and culture single cells by the use of glass capillaries realising single cell-based clonal analysis and the first description of a chemically defined medium [14, 38].

The early 1950s saw mammalian cell culture begin to develop a societal, cultural and ethical impact which would be felt through subsequent generations. This was marked by the isolation of the HeLa cell line from a carcinoma of the cervix and its subsequent emergence as a mainstay of cell research over the subsequent half century [39]. Shortly after this Harry Eagle defined the amino acid requirements for the L strain of mouse fibroblasts and in doing so provided the base recipe for many of the cell culture media formulations still in use today [6]. Progressive technological advancements were achieved over the next decade or so through improved instrumentation, engineering solutions and increasingly refined and defined media formulations. A good example of the continued ingress of engineering solutions was illustrated by the regulated cell culture incubator design presented by Ham and Puck in 1962 which in a similar manner to cell culture media contains many of the core design functionalities and construction parameters, including regulated CO_2 and humidity, found in twenty-first-century incubator design [19]. Simultaneous to his incubator design, Ham was also pioneering the formulation of new cell culture media describing initially his F7 and F10 solutions followed shortly after the description of the first chemically defined synthetic medium for culture of mammalian cells, F12 [20, 21]. At this point the broad consensus in mammalian cell culture was that all cells were principally immortal *in vitro* and that a failure to achieve continuous proliferation was due to technical failures in the culture environment. This dogma was overturned in the early mid-1960s by Hayflick and Moorhead with the pivotal observations of limited *in vitro* lifespans made in their descriptions of the isolation of foetal fibroblasts followed shortly after by the forming and advancement of the same hypothesis by Hayflick [23, 24]. The collected observations at this point have served to establish a firm foundation for cell culture research through to the current era. Additional key observations building on the frameworks established by Ham, Hayflick, Earle and Eagle amongst others include descriptions of the removal of serum from culture medium and its replacement by hormones and the observations that serum itself can induce premature mortality on mammalian cells which are bypassed by serum-free medium [25, 28]. These later observations necessitate an awareness that the cell culture milieu though well understood should not be viewed as complete but rather as a work in progress.

8.2 Oxygen in Cell Culture: Minimising the Shock?

At first glance the control of oxygen levels in cell culture appears to be a modern concern driven by an increased awareness of physiological approximation in model systems. However, it is quickly apparent that an awareness of the importance of controlling oxygen as a means to optimise culture conditions is evident almost as long as cell culture has existed. In the modern cell culture era, key reports date back to the late 1950s to early 1960s [5, 36]. The focus at this time was placed on oxygen toxicity and maximal tolerances vs. physiological normoxia effects. Collectively, Brosemer and Rutter, Rueckert and Mueller and Pace et al. as a component of their experimentation described the maintenance of a proliferative competency in a range of mammalian cells cultured in an N_2/5% CO_2 gaseous phase vs. toxicity at 95% O_2 [2, 32, 36].

By the mid- to late 1970s, an appreciation of a role of a physiological normoxia in mammalian cell culture had begun to emerge. Descriptions of enhanced clonogenicity, plating efficiency and cell growth of primary human fibroblasts had begun to emerge as it became increasingly clear that oxygen itself was a crucial component of the cell culture

environment [33, 43]. This was further evidenced by the observations that reduced oxygen concentrations increased the proliferative lifespan of normal human diploid fibroblasts including those first described by Hayflick [15, 24, 37]. The remainder of this section will discuss specific examples of the application of physiological normoxia to relevant cell types.

8.2.1 Methods and Apparatus

With over half a century of experience in minimisation of *in vitro* cell culture exposure to air oxygen, a number of strategies have emerged. However, it is important to note that air oxygen-based *in vitro* cell culture, irrespective of its lack of fidelity to *in vivo* normoxia, remains reflective of the vast majority of experimental reports, while the application of physiological normoxia would represent a very small proportion. Systems designed to replicate aspects of physiological normoxia *in vitro* include the traditional humidified CO_2 incubator modified to include N_2 gas as a displacement for O_2 from air levels, or ~18% [46], down to the desired levels. These, referred to as tri-gas, incubators can include additional glass door frontages intended to allow inspection of cells without loss of gaseous environment. The glass door frontage can on occasion be subdivided further into, e.g. 4, 6 or 8, separately opening access points again intended to minimise gaseous control loss to the external environment upon door opening. A further approach frequently adopted is the use of modular systems which are themselves then placed within standard incubators to be maintained at 37 °C. Examples of these include the Billups Rothenberg environmental chamber and the inexpensive inverted 1 litre container with screw top fastening [47]. In both these instances, the system is purged with premixed gas for a fixed spell before sealing and placing into the standard 37 °C incubator. Commercial variants of these systems with increased sophistication can include real-time gas monitoring and modulation. The immediate drawback of the systems mentioned above is that all require handling outside of the controlled environment for microscopic viewing, media changes and passaging. For each of these steps, a loss of the desired gaseous environment and exposure to room air are an inevitable consequence of the action. In the tri-gas incubator, this loss of controlled gaseous environment will impact on all cells within the incubator, whereas the modular system will only impact on those stored within. Routine media changes and passaging would be performed in a standard class II biological safety cabinet again causing exposure to air oxygen. The situations described above represent the bulk scenario of most *in vitro* physiological normoxia cell culture experimentation.

Continuous gaseous environmental control through imaging, media changes and passaging can be provided by adoption of a hermetic chamber. These units, frequently with front glove ports for access and separate materials entry ports, act both as incubators and workstations providing controlled humidity, temperature and oxygen level control and in doing so remove the unavoidable fluctuations in oxygen experienced by the incubation methods described above. Limitations to these systems include difficulty in access, restrictions to usage via multi-user limitations and high gas usage. The final, and frequently overlooked, component of oxygen control is that of the medium itself. Nitrogen bubbling and passive deoxygenation have been described as measures to reduce the oxygen content of cell culture medium before its exposure to cells though these measures are difficult to control, variable in effect and not effective at scale. More recently we have developed the HypoxyCOOL™ system in collaboration with Baker Ruskinn. The HypoxyCOOL™

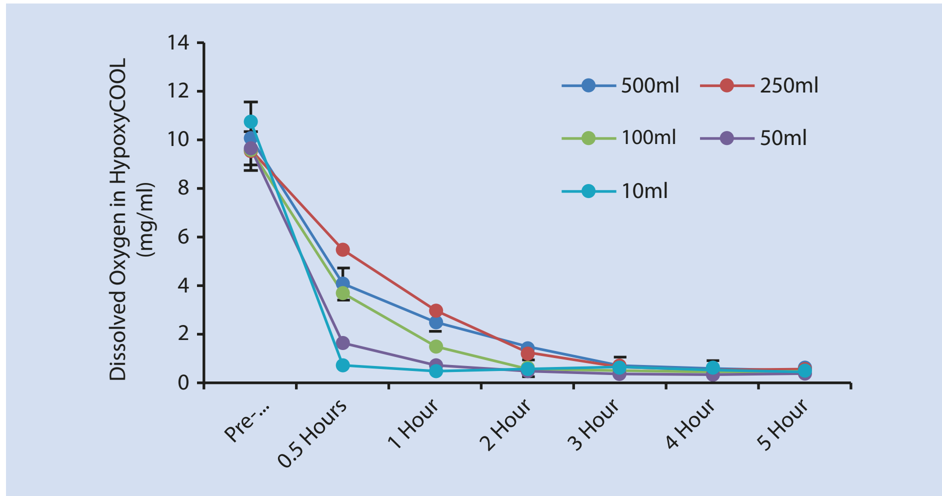

Fig. 8.1 Defined deoxygenation of cell culture medium with HypoxyCOOL™. Different volumes of DMEM medium were placed into the HypoxyCOOL™ unit and deoxygenated over a range of times (hours). DMEM bottle caps were removed aseptically and replaced with 0.2 μm vented caps before placing into unit. Smaller volumes were deoxygenated in 15 ml or 50 ml sterile tubes. Headspace was defined as 1% O_2, 5% CO_2 and 94% N_2 and cycle time as specified on the *x*-axis. Measurements were performed with the Microx 4 Microsensor (Presens, Germany). All data points represent $n = 3$. Error bars indicate standard deviation. *X*-axis indicates time (hours), and *y*-axis indicates dissolved oxygen (mg/ml)

system combines controlled agitation, refrigeration and a defined headspace to deoxygenate culture media rapidly to desired levels (Fig. 8.1). The combination of workstation technology alongside a specified, deoxygenated, medium generates a platform for the provision of a controlled physiologically normoxic environment. At this time there are limited options available allowing empirical, simultaneous *in vitro* experimentation across multiple oxygen levels to allow definitive optimisation of individual cell type normoxia requirements.

8.2.2 Pluripotent Stem Cells

The remainder of this chapter will discuss instances where physiological normoxia, or best approximation, has been applied to two clinically relevant cell types: pluripotent stem cells (PSC) and multipotent bone marrow-derived mesenchymal stem cells. Pluripotent stem cells have an inherent capacity to differentiate into all three germ layers being first described in 1981 for mice and 1998 for human [12, 30, 44]. A role for physiological normoxia was first proposed for human embryonic stem cells in the mid-2000s with a collective description emerging which described reduced spontaneous differentiation, enhanced clonogenicity, reduced chromosomal aberrations, a role in optimised de novo line isolation and a substantially altered transcriptome (vs. air cultured cells) [13, 16, 17, 29]. Where information is specified, it is clear that these studies drew on modular systems (see ▶ Sect. 8.2.1) for provision of physiological normoxia (in the range of 1–5% O_2) with handling (media changes, passaging) in air oxygen. The PSC repertoire underwent a significant and substantial expansion in 2006 with the first description of the generation of induced PSC from

mouse fibroblasts and a year later from human [41, 42]. Subsequently it was demonstrated that the generation of reprogrammed iPSC from both human dermal and mouse embryonic fibroblasts could be substantially enhanced by incubating samples in a tri-gas incubator held at a 5% O_2 level [48]. As above all manipulation steps were performed in air oxygen. As is clear from the above highlighted studies, it remains to be determined what additional role, if any, a controlled physiological normoxia environment where repeated air oxygen exposure is removed would have on PSC biology though this is identified by several authors as a potential limitation of their studies.

8.2.3 Multipotent Bone Marrow-Derived Mesenchymal Stem Cells

Pioneer descriptions of what have become known as bone marrow-derived mesenchymal stem cells (MSCs) date back to the late 1960 through Friedenstein's early transplantation experiments [18]. Repeated *in vitro* experimentation culminated in the 1991 hypothesis by Caplan of the existence of an *in vivo* MSC with a multipotent, mesengenic, differentiation capacity and the subsequent 1998 description of these cells by Pittenger et al. [3, 34]. Minimally hMSC are expected to have a tripotent differentiation potential: bone, fat and cartilage. With a history spanning back over the last half century, vs. the previous 20 years for hPSC, and relatively straightforward isolation and culture, it is unsurprising that hMSC have a vast accompanying literature as well as a high therapeutic potential evidenced by multiple, global, clinical trials and a surrounding bioscience industry [45]. A wide range of physiological normoxic conditions have been applied to the isolation of hMSC from bone marrow with a reported range spanning from 1% to 5% O_2 [27]. Importantly, and unlike hPSC, these studies have begun to incorporate hermetic workstations allowing continuous exposure to a controlled physiologically normoxic atmosphere resulting in enhanced cell isolation in comparison to air oxygen exposure and subsequent accumulation of oxygen 'shocks'. Intriguingly there are reports of isolation of hMSC for clinical application under 'hypoxic' conditions, 1% O_2, though it is unclear how these conditions were achieved, i.e., tri-gas, modular or workstation incubation [1]. It is clear therefore that substantial steps have been taken with hMSC and the application of physiological normoxia from research through to clinical usage readiness. However, similar to hPSC the incorporation of defined medium oxygen control into these hMSC isolation and characterisation measures has yet to be undertaken and outcomes presented.

8.3 Future-Proofing Cell Culture: Creating a Normoxic Model

In vitro cell culture has come a long way from its earliest descriptions and practitioners through to its mid-twentieth-century pioneers and into the modern era. Many aspects of the modern cell culture environment remain virtually identical to those first developed by the band of pioneers highlighted in ► Sect. 8.1. It is now becoming increasingly clear that generating model environments reflective of the *in vivo* environment are essential for improved understanding of cell biology and behaviour as we move forward into the cell therapy era. Physiological normoxia is a single component of the environment, and equal attention will be required of sugars, salts, serum and matrix, amongst others.

Technology has begun to emerge which can now provide continuous control of the oxygen environment. This will help move the field beyond the tri-gas incubator and gas-flushed modular systems into controlled hermetic workstation and controlled media oxygenation approaches. New technologies will likely emerge to provide improved handling and transportation while combining the added value of improved experimental control in a continuously physiologically normoxic environment.

Take-Home Message

- Mammalian cell culture has a rich century-old history.
- Physiological normoxia experimentation in mammalian cell culture dates back to the mid-1950s.
- Technological advances have generated state-of-the-art equipment which can now provide continuous oxygen-controlled environments for *in vitro* cell culture.

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Co-cultures

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What You Will Learn in This Chapter

Monocultures of cells including the study of their behavior at distinct conditions (pH, ionic strength, anoxic, hypoxic, normoxic, hyperoxic, etc.), their activation/stimulation with different agents (cytokines, chemokines, growth factors, metabolites, drugs, etc.), or their differentiation along certain pathways within the three germlines provide a facilitated model to obtain general answers. However, natural cell behavior, activation, or differentiation in fluids and tissues is much more complex involving three-dimensional interactions with several different cell types in the local vicinity and microenvironment rather than a two-dimensional development as an isolated cell type in a culture dish. Therefore, the study of cellular behavior in the presence of other cell types and their multiple cellular interactions requires the establishment of corresponding co-culture models.

Accordingly, this chapter will guide you through the complexity of cellular co-culture systems, e.g., considering indirect (transwell) co-culture which allows the exchange of common medium- and small-sized cellular or soluble products. In addition, this chapter will also provide various hints for a direct co-culture of different cell populations, starting with an appropriate cell ratio of the monocultures and finding the right culture medium for both cell types without changing their functionality. Moreover, examples are given for labeling the different cell populations to distinguish them in co-culture, and finally, certain alternative assays are suggested for discrimination of potential functional differences within the distinct cell populations during co-culture.

9.1 Culture Techniques

9.1.1 Direct Co-culture

9.1.1.1 Adherent Cells and Nonadherent Cells

Culture conditions depend on various parameters specified by the needs of the particular cells wished to keep in culture. Basically, cells can grow either adherently or in suspension. Moreover, cell culture needs to be distinguished between primary cell populations and cell lines. Usually, primary cells display a limited life span, whereas cell lines exhibit properties of unlimited growth.

After seeding, cells suitable for in vitro expansion will go through a defined growth cycle. The cycle starts with a lag phase before the cells proliferate and is followed by the exponential, or log phase, when the cells are rapidly dividing and the population continuously doubles for a certain time. Finally, the cells enter the plateau phase which is initialized by several factors such as reduced nutrient or substrate availability and contact inhibition which limits further cell proliferation. As a result, adherent cells will finally form a confluent monolayer. Although cells in suspension do not necessarily get in physical contact, they normally require a cell-specific density, below and above which cell growth is suboptimal.

According to these culture parameters, four different conditions may result from a co-culture system whereby certain effects need to be considered:

1. Co-culture of adherent primary cells with an adherent cell line → the adherent cell line may eventually overgrow the primary cells; however, the co-culture may be limited by confluency.

2. Co-culture of adherent primary cells with a suspension cell line →the suspension cell line can easily overgrow the primary adherent cells, since suspension cells do not reach confluency but may be limited by density.
3. Co-culture of suspension primary cells with a suspension cell line →the co-culture will be limited by the cell density of both cellular partners.
4. Co-culture of suspension primary cells with an adherent cell line →the co-culture may be balanced, while the primary cells in suspension are limited by cell density in the supernatant and the adherent cell line is limited by the available space until confluency.

Co-culture conditions and the time scaling of experimental setup also depend on differences in the proliferation rate and cell cycle progression of each of the cellular partners. Studies on cellular interactions between different cell populations also require determination of the initial seeding number of the co-cultured cells, particularly if the corresponding cell partners display significant differences in their proliferative capacity. For example, cell type A with a 24 h doubling time and cell type B with a 48 h doubling time would be co-cultured ideally with a majority of cell type B compared to cell type A at the initial co-culture start to limit rapid overgrowth of cell type A [14].

9.1.1.2 Feeding (Requirement of Centrifugation, Sedimentation, Detachment, Sorting)

As long as the cell density remains below maximum, all environmental parameters have to be maintained optimal, for example, by changing media. This provides sufficient nutrients and factors needed for cell proliferation and also ensures the essential removal of metabolites and waste material from the media. In addition, metabolic activity also affects the pH value of the media which is a critical factor for most cells. Therefore, in general, the vast majority of the media is buffered to stabilize the acid-base status, and in addition, many contain indicators such as phenol red which simplifies pH value control by color change (e.g., from red to yellow in case of acidification). In case of adherent cells, media exchange can be performed by partly or completely decanting the supernatant and replenishing the culture with the corresponding amount of preheated fresh media. In contrast, cells growing in suspension require sedimentation, for example, by centrifugation prior to media exchange.

An important requirement for cellular co-culture is the optimal growth medium. Usually, each cell type is growing at individual conditions in a certain kind of medium supplemented with distinct growth factors which most likely do not match between the co-culture partners. Therefore, preliminary experiments are recommended with different mixtures of the corresponding growth media of the two co-culture partners to evaluate optimal growth conditions for both cell types. Such preliminary tests also enable the determination of proliferative capacity of each co-culture partner in the optimized co-culture mixed medium.

Once the cell density reached its optimum, cells have to be split. To this end, cell suspensions can be aliquoted by transferring a suitable number of cells into fresh media. In contrast, adherent cells usually need to be detached from the culture dish surface they are growing on prior to splitting. This can be facilitated by physical or biochemical techniques. After media exchange with an isotonic serum-free solution such as phosphate-buffered saline (PBS), Hank's buffered salt solution (HBSS), or saline and subsequent addition of

proteolytic enzymes (e.g., trypsin, accutase), cells are incubated for a few minutes until the cells are fully detached. The supernatants containing the cells are harvested, and enzymes are inactivated by adding serum containing natural antitrypsin activity which also serves as competing protein substrate. In order to prevent proteolysis of cell surface proteins, cells can also be detached by using cell scrapers, which, however, might physically damage the cells. Separation of the co-culture into the corresponding monocultures is simple if cells of different adherent properties are cultured such as adherent cells with suspension cells. In case of homotypic properties (both cell partners are suspension or adherent cells), a separation is much more difficult. For this purpose, the mixed co-culture can be separated, for example, by magnetic bead-associated cell sorting (MACS technology) according to a specific cell surface marker of one of the cellular partners. Alternatively, the cells can be labeled by a fluorescent dye (e.g., GFP or mCherry) prior to the co-culture, and separation into the respective monocultures can be performed by fluorescence-activated cell sorting (FACS) following co-culture [12].

Independent from the kind of detachment used for harvesting, inspection of cell viability is always required prior to performing experiments and subcultivation. A suitable way to check for dead cells is, for example, facilitated by mixing the cells with trypan blue dye, which permanently stains the cytoplasm only of damaged cells that can then easily be detected by microscopy.

9.1.1.3 Shape of Dishes and Plates (Culture U-Bottom, V-Bottom, Flat-Bottom)

In order to enable cell to cell contact, cells can be cultured in appropriate dishes or plates. Standard culture containers are flat, round (U)-, and pointed (V)-bottom tubes or wells in culture plates, respectively. Flat bottoms are useful for studying morphology and motility of cells by inverted microscopy without distortion by light refraction. U-bottoms are less suitable for microscopic monitoring. However, the U-shape sediments suspended cells gently, enabling soft cell contact, thereby supporting cell to cell signaling and physical interactions. Compared to U-bottom, sedimentation is increased when cells are maintained in V-bottom containers by which the cells are stronger packed. In addition, V-bottoms allow easy and efficient harvesting of cell-free supernatants. In contrast to other shapes, V-bottoms are not suitable at all for inverted microscopic inspection [7].

As outlined above, a certain cell to cell ratio is employed, which depends on several parameters that provide optimal conditions for the specific experiment. The ratio could, for example, be deduced from physiological conditions present at natural site of interaction in the body. It could also be based on theoretical considerations in order to achieve a certain effector to target ratio, for example, in case of cell-based tumor therapies.

A very important factor is the duration of the co-culture, particularly if the partners exert significantly different growth kinetics. The fast-proliferating cell population might then overgrow the less dividing cell population shifting the cell to cell ratio toward one competitor. Assuming that doubling of mammalian cells in culture takes about 10–12 hours it seems reasonable to not extend co-culture periods too much. Depending on cell type (e.g., tumor cells, primary cells) and other parameters (e.g., provision of growth factors), the reasonable duration of the co-culture has to be determined prior to any kind of experiment or analysis.

Another essential factor for a successful co-culture is the mutual tolerance of the employed cells. While normal somatic cells tolerate each other even if they were originally derived from different individuals (allogeneic) or species (xenogeneic), immunocompetent cells could target co-cultured cells. Targeting means recognition and responding to the challenge. The response patterns of the immune cells depend on their specific capabilities and could result in increased proliferation, release of soluble factors (cytokines), but also killing of the target cells.

Induction of proliferation requires recall antigens present on the stimulating cell and specific receptors on the responder side when purified responder cells are employed. When PBMC instead of purified lymphocytes are used, no cognate antigen is necessary to induce proliferation. PBMC also comprise antigen-presenting cells (APCs) such as dendritic cells (DCs) and monocytes, which can sense foreign antigens present on the stimulators. APCs take up, process, and present the antigen to the responders, thereby inducing their proliferation. In this case, the co-culture will so to speak mimic a classical immune response and is known as mixed lymphocyte reaction (MLR). MLR could be used to test in vitro tissue compatibility between donor and recipient in a transplant setting.

Other responder cells could kill stimulator cells. In this case, stimulators must be equipped with a cytolytic apparatus which enables the killing of the stimulating cells. Main killing mechanisms are based on direct lysis by disintegrating the cell membrane and/or by inducing programmed cell death (apoptosis). To this end, the effector cells release preformed cytolytic molecules from intracellular granules like perforin and granzymes. Perforin forms pores in the target membrane and enables granzymes to enter the cell and destroy the nucleus finally causing cell death. However, apoptosis of a target cell can also be induced by directly activating death receptor containing surface proteins by the effector cell which induces apoptosis bypassing prior cell membrane destruction [15].

However, also nonimmune cells can sense and respond in syngeneic and allogeneic settings. For example, mesenchymal stem cells (MSCs) can affect functions of co-cultured cells via soluble factors as well as direct cell to cell contact. MSCs are fibroblast-like cells that can be isolated from different sources such as adipose tissue, peripheral blood, the lung, and the heart [4]. MSCs have the potential to develop at least along osteoblast, chondroblast, and adipocyte lineages [5]. In addition, they exert a strong immunosuppressive capacity which can be mediated by soluble factors like prostaglandin (PG)E2 and activin A as well as direct cell contact. However, the receptor ligand pairs required for establishing the contact and enabling signal transduction are not identified so far [1]. It is possible that the close physical proximity between the cells alone is sufficient for signaling even if the soluble factors are released at very low concentrations. Close proximity of the cells is, for example, also essential for the immunosuppressive mechanism based on the expression of the ectonucleotidases CD39 and CD73. Expressed on the surface in suitable density, they collaborate in degrading ATP in the environment via AMP to free adenosine. Adenosine binds to adenosine receptor which blocks phosphorylation of intracellular activating molecules such as ZAP70. This mechanism requires the availability of the substrates ATP and AMP in sufficient amounts in order to cleave off free adenosine. In addition, the distance between ectonucleotidase-expressing cells and responder cells in the co-culture needs to be short enough to provide a sufficient local concentration of free adenosine [2].

9.1.2 Transwell System

9.1.2.1 Contact-Dependent Interactions, Effects of Soluble Factors

In order to investigate if cellular interactions require cell to cell contact or are mediated by soluble factors, transwell systems can be employed as an appropriate tool. Basically, a transwell system comprises a normal cell culture dish containing one cell type and an insert with the other cellular partner (Fig. 9.1). The bottom of the insert is a membrane, which is permeable for all soluble factors but not for cells. Accordingly, membranes of different pore sizes (>0.2 μm) are available for transwell co-cultures to allow/prevent exchange of culture medium and factors/particles of certain size. Cells from both containers can be analyzed independently, and any co-culture-induced effect must be contact-independent and soluble factor-mediated since the cells are physically separated while they share the same media. However, it has to be taken in consideration that the distance between the factor-releasing cell and the responding cell might be too far and thus a low factor concentration may not suffice to exceed the threshold necessary for its activity. Moreover, formation of a local threshold of metabolites with reduced diffusion coefficient may limit the effectiveness to sufficiently induce target cells. Furthermore, short-lived metabolites also exhibit limitations to address adequate signals in the target cells which, however, would be more likely observed in a direct co-culture and, therefore, may include the risk of a wrong interpretation of a direct co-culture dependency. Consequently, transwell experiments need to be carefully designed, and resulting data require double-checking by appropriate control experiments to justify any conclusions [3].

Analyzing co-cultured cells apart from each other might require re-isolation techniques which depend on the specific cells that were employed. From transwell systems, both cell partners can be simply harvested since they have not been mixed up. A suspension of different cells cultured in the same dish is more complicated to separate afterward from each other. If one partner is adherent, while the other is growing in suspension, the isolation can be easily performed by harvesting the supernatant to recover the nonadherent cells and detaching the adherent cells from the dishes.

In case of two adherent cells or two cell types growing in suspension, the two partners finally have to be sorted out from each other prior to further analysis. This can be done, for example, by microscopy if cell properties (e.g., the morphology) significantly differ from each other and readout parameters can be visually evaluated. For a more detailed analysis, flow cytometry can be considered. Usage of one characteristic distinguishing marker for each of the two partners enables determining other intra- and extracellular markers separately for both cell populations. If this virtual cell separation is not adequate because further functional experiments are contrived, the cells have to be physically separated, for example, by flow cytometric or magnetic sorting (see above). Here, we don't want to go too much into detail, but it is important to adequately plan the sorting strategy. Both methods are based on specific antibodies which are either labeled with dyes (flow cytometric cell sorting) or paramagnetic particles (magnetic cell sorting). Depending on the available flow cytometric sorting device and hence the number of fluorescence detectors, several population might be simultaneously sorted from one cell population. In addition, it is also possible to sort according to the expression density of a molecule (e.g., low and high expression). In contrast, magnetic sorting normally just allows the discrimination of two

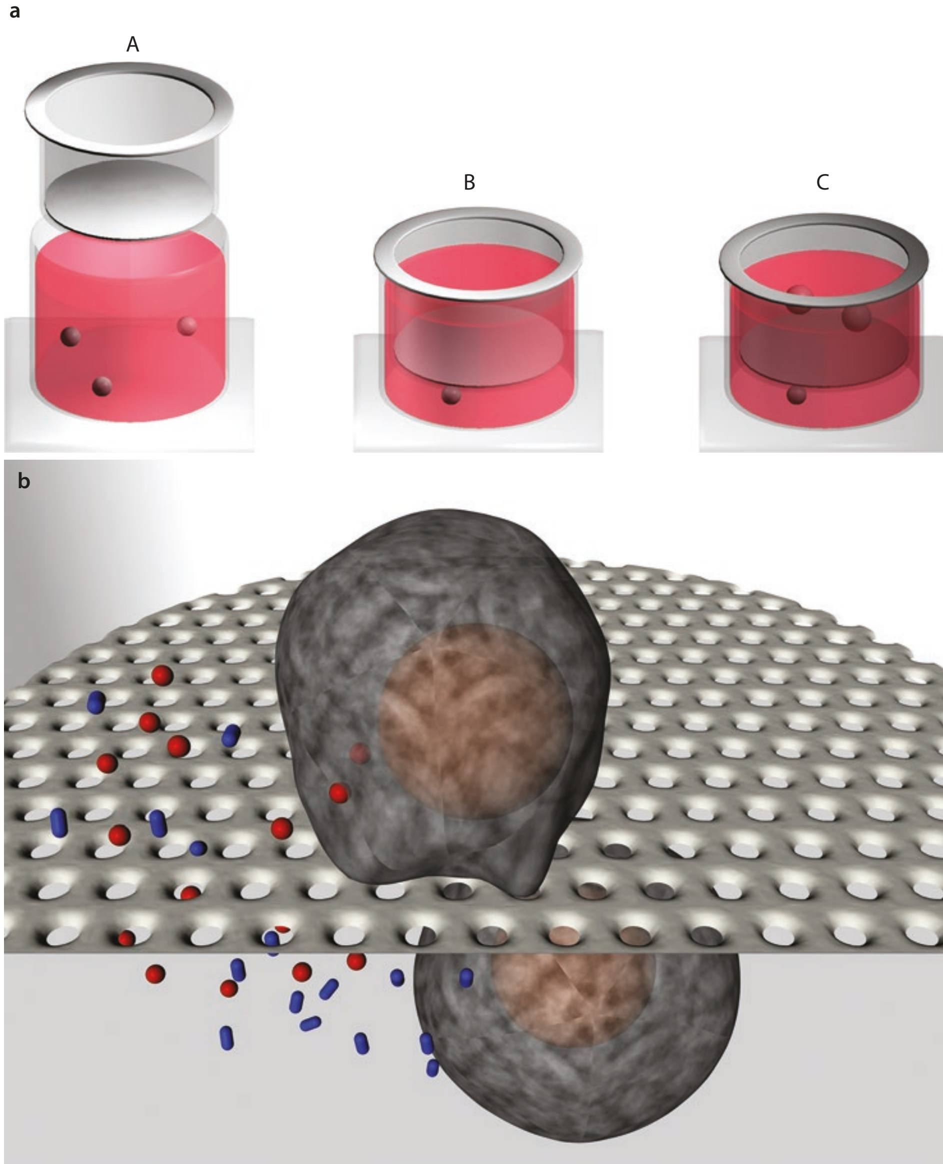

Fig. 9.1 Transwell culture system. **a** Cells are cultured in a dish (A). An insert with a membrane is placed into the dish (B). Different cells are loaded into the media on top of the membrane (C). **b** The pores of the membrane are too small to enable any contact between cells of both chambers while media and soluble factors (red and blue particles) can freely spread between the two compartments

conditions (positive or negative) but requires less sophisticated equipment. Independent of the method used, a possible activation of the cells by using antibodies for cell sorting has to be considered. The antibodies might bind surface receptors resulting in cell activation signal by cross-linking. Therefore, a negative sorting strategy should be preferred if possible, meaning that the unwanted cells are targeted by antibodies and removed while leaving the cells of interest untouched [13].

9.1.2.2 Migration Assay (Boyden Chamber)

While transwell systems have very small pores that prevent cells from passing through, migration systems like Boyden chambers are equipped with wider pores enabling cells to migrate through the membrane if the attracting stimulus is sufficient. The pore diameter of an adequate membrane depends on the subject cells. For example, 3 μm pore size is appropriate for leukocyte or lymphocyte migration, and 5 μm pore size might be suitable for monocytes and macrophages. Pores of transwell systems normally have diameters of 0.4 μm.

9.1.3 Culture Medium

9.1.3.1 Optimal Medium Composition and Conditions for Co-cultures

Growth conditions depend on various factors such as environmental temperature. While, for example, insect cells can be maintained at room temperature, standard temperature for culturing mammalian cells, which will be focused on hereinafter, is 37 °C. However, the optimal temperature might be slightly different as derived from physiology of the respective source species. Accordingly, incubators can be adjusted to the cell-specific optimal temperature and regulated by rheostat control.

9.1.3.2 Gas Conditions (Normoxia, Hypoxia, Anoxia)

Gas exchange between cells and environment is another fundamental factor for growth conditions. Gas exchange is essential for cell breathing but also for stabilizing the cell environment. Efficacy of the exchange strongly depends on partial gas pressure and ventilation of the incubator and also the employed dishes. For this reason, culture flasks are also available with caps equipped with gas-permeable membranes.

While the normal $C0_2$ concentration of tidal air is around 0.04%, the atmosphere in incubators is enriched with additional CO_2 reaching levels of 5% or even more. However, the additional CO_2 is not provided in order to improve cell breathing but rather to stabilize pH value of the media. Generally, in order to provide optimal culture conditions, the media in which cells grow need to be buffered to achieve in most of the cases more or less neutral pH (around pH 7). Sodium phosphates, Tris or Hepes, can be added as buffering compounds, but these agents might also negatively affect the cells' growth. The HCO_3- and H_2CO_3 carbonate buffer system has been found to affect the growth of cells the least. H_2CO_3 in water is in equilibrium with $H_2O + CO_2$, and in order to keep the CO_2 in the buffer solution at the right level, CO_2 has to be supplemented to the atmosphere above the buffer. Otherwise, all the CO_2 would be deprived, and thereby the H_2CO_3 be withdrawn, disabling the buffering effect (the medium would become alkaline).

Cellular respiration means specifically the gas exchange by oxygen uptake and CO_2 elimination and is essential for any aerobic cell. Dry air contains around 21% oxygen, while in tissues and plasma, the physiological environment of cells, oxygen concentrations could considerably differ. The actual oxygen condition which might be higher, equal, or lower compared to the air is referred to as normoxia, hypoxia, and anoxia. Hyperoxia exerting higher levels (>21%) of oxygen plays no role in cell culture systems as it is normally not occurring and would be toxic to cells. Oxygen tensions between 10 and 21% are described as normoxia. Culture of cells or tissues at lower oxygen levels can be achieved by addition of variable amounts of nitrogen to the incubator which replaces

oxygen and thereby lowers the O_2 concentration. For this, the incubator requires a special equipment including an O_2 electrode to equilibrate to the desired oxygen concentration. Alternatively, specific hypoxia chambers can be used for variation of O_2 levels. Hypoxia is a condition of low oxygen, typically in the range 1–5% O_2, whereby even lower O_2 levels are considered as anoxic conditions. This is often observed in central regions of tumors due to poor vascularization. Clearly, the prevailing O_2 concentrations affect cell growth, and particular experiments may require variation of the O_2 tension in order to achieve special conditions.

9.1.3.3 Evaluation of Growth Properties and Functionalities (Cell Surface Markers) in the Adapted Co-Culture Conditions

Over time, cells may alter in culture which could, for example, result in changing morphology, marker profiles, functional capabilities, viability, and genetic stability. Hence, the different features have to be regularly controlled during maintenance. Cell morphology can be easily assessed by inverted microscopy of the culture in the dish. Alternatively, cells can be taken from the culture and scrutinized by light or fluorescence microscopy, respectively. Determining the marker profiles requires staining of a cell aliquot with appropriate antibodies prior to subjecting the sample to flow cytometry or comparable methods. Cell viability can also be assessed by light microcopy using trypan blue staining solution or flow cytometry using nuclear dye such as propidium iodide (PI) or 7AAD. Trypan blue can pass damaged cell membranes only, indicating respective cells as dead by intense blue staining of plasmatic proteins. Similarly, PI and 7AAD cannot permeate intact cell membranes. Once the cell membrane is disrupted, the dyes can bind to the nucleus resulting in clear staining of dead cells only. By combining this analysis with determining annexin V exposure, it is possible to evaluate the degree of early apoptosis which precedes cell death. A very variable feature of cells is their functionality and depends on their specific genetic capabilities. These functions could be the ability to migrate, move, contract, conduct stimuli, generate or conduct electricity, pulse, produce and release metabolites, kill other cells, or phagocytose particles or pathogens. For most of these functional features, appropriate assays are available and could be employed to monitor for changes over time. During in vitro culture, DNA repair mechanisms of the cells may not be as effective as in the living organism. Hence, genetic stability of the cells may be less and the risk of mutations could be increased. If occurrence of culture-induced mutations is suspected, monitoring by PCR or sequencing techniques could be useful [8].

9.2 Cell Properties

9.2.1 Long-Term, Short-Term Culture and Long-Lived, Short-Lived Cells

Changes over time have to be taken into consideration especially if cells are kept in culture or co-culture for a longer period. Short-term cultures for up to 24 h hours are in most cases not too much affected by the aspects mentioned above. However, this also depends on the cell type which is employed. There are long-lived as well as short-lived cells. To illustrate this, white blood cells may serve as an example. While lymphocytes from peripheral blood can be cultured in vitro without any problems for weeks, in contrast, most of the granulocytes from the same sample will be dead after 24 h.

9.2.2 Reactivity (Stimulation, Sensitivity to Killing)

Success of a co-culture is also dependent on compatibility of the employed cells. In most of the cases, different cell types are compatible, which means they can be cultured together without affecting each other. For example, co-culturing mouse muscle cells and human nerve cells will be mostly harmless for both cell types. However, co-culture of immune cells could have significant effects on one or the other cell type. The outcome depends on the multifaceted ability to sense each other. Both cells might ignore each other due to lack of any cognate receptor ligand pairs. However, one cell might recognize its counterpart. This could, depending on the cellular capabilities of the two involved cells and their receptor and ligand equipment, respectively, result in activation of one, the other, or both cells through receptor/ligand triggering. Indeed, the ligand/receptor interaction could also lead to agony or tolerance. Furthermore, recognition of one cell could also induce destruction of the co-cultured cell. This could happen if the effector is a cytotoxic cell that senses a target cell (e.g., virus infected or tumor cell). It is obvious that these interactions, which will be explained below in more detail, significantly affect the co-culture system (see Intended Role of the Cultured Cells) [10].

9.2.3 Cell Origin

Growth kinetics and dynamics are affected by the cell source. Primary cell cultures are initiated by subjecting cells to culture directly after isolation from fresh blood or tissue samples. To initiate a primary cell culture can be difficult and may require much effort to define suitable culture conditions. After subculturing, the cells are termed secondary cells. In case of adherent cells, a subculture requires prior detachment by extracellular matrix protease treatment and seeding in an adjusted cell concentration which is termed passaging of the cells, and the passage number of the subcultured cells is increased accordingly. Maintaining secondary cells is easier than primary cells since culture circumstances are already established and cells might also be adapted to in vitro conditions. In most cases unproblematic is the culturing of cells obtained from tumors or artificially immortalized cells, respectively. These cells are characterized by an unlimited division capability providing hereby an inexhaustible source of cell material. Primary cells are closest to normal in vivo conditions, while secondary cells might already have undergone alterations in their genetic and phenotypical profiles.

Immortalized cells are unnatural and might considerably differ from the originating cells in all aspects, which have to be taken in account when using these cells for experiments. Most cells, regardless if primary, secondary, or tumorous, can be cryopreserved until usage. To this end, cells are stored in liquid nitrogen after resuspension in freezing media containing dimethyl sulfoxide (DMSO) as cryoprotectant. DMSO prevents the formation of intracellular ice crystals that would otherwise destroy the cells. Nevertheless, cell recovery after thawing is significantly less than 100%, and the cells are stressed by the freezing/thawing procedure. Therefore, cell growth and function can be delayed after thawing and might require some extra time to regain full functionality.

9.2.4 Cell Compatibility

If the different cells intended for co-culture stem from the same individual, the system is termed autologous. Since all cells have an identical makeup, no cellular sensing and challenging will occur in this setting. Even if immunocompetent cells are included, immune responses will not occur since immune cells develop under maturation pressure, which ensures that any potential autoreactive cells will be eliminated immediately after emergence.

Allogeneic means that cells of two or more individuals of the same species are involved. In contrast to protozoa, some lower species, and inbred animals, cells of all higher animals express individual patterns of surface proteins genetically encoded in the so-called major histocompatibility complex (MHC). These proteins are marked by a very strong individual variability making the individuals of a species unique and different from each other also on a cellular level. These individual patterns are of particular interest in cell and organ transplantation since disparity could result in graft rejection. A strong mutual activation of immune cells in a mismatched allogeneic co-culture is very likely, while nonimmune cells of the same donors can be co-cultured without any interference.

In a xenogeneic system, co-cultured cells stem from individuals of different species. This combination is difficult to envisage regarding the response of the cells exposed to foreign cells. Most probably, most cells of different species will not interfere with each other. In case of immune cells, the response patterns will mostly depend on the ability to sense foreign molecules on the co-cultured counterpart. However, like in the allogeneic system, activation of immune cells by xenogeneic stimulators is very likely.

9.3 Cell Ratios

When determining the optimal cell ratio for co-culture experiments, it has always to be considered that cells optimally grow and function at a certain specific cell density. Therefore, first, the optimal individual cell density has to be determined and then the ratio required for the particular experiment. If intended to mimic in vivo conditions, the ratio may be selected according to the in vivo conditions where, for example, type x cells may be ten times more than type y cells. In that respective experiment, a certain number of type y cells would then be co-cultured with ten times more type x cells. However, in vitro conditions cannot fully adapt any in vivo situation where cells are organized in tissues in which they reside and can also actively migrate. Different types of cellular movement can be distinguished including a slower mesenchymal mode of migration which is exhibited by fibroblasts or mesenchymal stroma/stem cells by strong substrate adherence, prominent stress fibers, and extended formation of protruding lamellipodia/ruffles at the leading edge. Alternatively, a faster amoeboid-like cell migration is utilized, e.g., by immune cells, and characterized by weaker adhesion, absence of stress fibers, and formation of action-rich pseudopods in the cellular fronts paralleled by myosin II-driven contractility in the rears. Migration is not a random process but mostly driven by gradients of chemicals such as chemokines which are released by other cells in the environment. By this mechanism, susceptible cells are recruited to the site of the highest chemokine concentration where the cells can then interact at a locally and temporarily much higher ratio between the

interaction partners as compared to other areas of the same tissue. Since it is nearly impossible to simulate the complex cell interactions found in vivo, it is indispensable to find out the optimal cell ratios by trial and error for every experimental setting. Clearly, reasonable ratios have to be selected to give the cells a real physical chance for interaction.

9.4 Intended Role of the Co-cultured Cells

The intended role of each cellular partner in a co-culture experiment is of particular importance for planning the culture system. The employed cells could, for example, serve as stimulators, responders, feeder cells, target cells, competitors, transporters, and supporters.

9.4.1 Mixed Lymphocyte Reaction

Using cells as stimulators or responders is typical for mixed lymphocyte reactions (MLR) or mixed lymphocyte culture (MLC). In this case, PBMC of two different individuals are co-cultured for a few days, and proliferation is determined. This culture setup is called a two-way MLR as it allows both partners to respond to the stimuli offered by the other one. This means both cell fractions act as stimulators and responders at the same time. In contrast, in a one-way MLR, the proliferative capability of one cell suspension is prevented, for example, by irradiation. These cells can act only as stimulators, whereas the nonirradiated cells can respond with proliferation. MLR is a suitable assay to determine the foreignness of two individuals and hence useful to check for the ability of rejection in transplantation settings.

9.4.2 Cytotoxicity Assay

Cellular cytotoxicity assays also involve at least two partners which are called in this context effectors and targets instead of responders and stimulators, respectively. Principally, this assay is a short-term co-culture of effector and target cells. The effector cells are capable to lyse susceptible target cells, whereas the latter are not. Therefore, the targets don't have to be inactivated or irradiated prior to the assay. At the end of the assay, the percentage of dead cells are determined, for example, by measuring release of radioactive isotopes or using fluorochromes in flow cytometry. An important feature of this kind of assay is the selection of adequate E/T ratios. The standard natural killer (NK) cell cytotoxicity assay might serve as an example. This assay is normally performed by using different effector (E)/target (T) ratios spanning a range in which reliable results are expected. In the human system, for example, radioactive chromium-labeled K562 cells are used as targets. K562 is a human tumor cell line which lacks MHC-1 and is therefore susceptible only to NK cell-mediated killing. If pure NK cells (NK cell lines, clones, or sorted NK cells) are employed as effectors, E/T ratios of 10:1 or 5:1 will be optimal for lysing approximately 20 to 80 percent of the tumor cells as assessed by measuring the release of radioactive chromium. If PBMC are used instead of pure NK cells, the ratios have to be adapted in order to achieve comparable results. Since PBMC roughly contain 15% (one sixth) NK cells, six times higher E/T ratios such as 60:1 and 30:1 are used. Many researchers also include further E/T ratios (e.g., 15:1 and 7.5:1) since in most of the cases the exact number of NK cells is not known when the assay is started [6].

9.5 Co-culture Manipulation

Above, it is shown that the function within a cell mixture might be attributed to only one partner. This can be due to the natural different functional capabilities of the co-cultured cells (e.g., effectors can kill; targets cannot) or by inactivating one cell population, e.g., by irradiation. Further techniques for manipulating cell suspensions can be employed in co-culture. Sometimes, it is wanted to eliminate defined cell subpopulations. This can effectively be facilitated by depletion using specific antibodies and respective fluorescence- or magnetic-based techniques. If the presence of a particular cell in a co-culture is irrelevant, however, the interaction with other cells is aimed to be suppressed; it might be suitable just to eliminate specific functional properties. This could be facilitated by blocking receptors or factors released by the cells by using small chemical inhibitors or blocking antibodies. It is also possible to directly suppress the expression of specific molecules, for example, by treating the cells with siRNA. On the other side, to investigate the importance of a particular molecule, it might be suitable to increase the expression of particular molecules by adding, for example, cytokines or by genetic transfection of the cells. In this regard, it is always to be considered that in most co-culture systems several different effects are induced. The stronger effects might then overweigh weaker signals making them invisible and become only apparent when the main effects are blocked. For example, in a recent study, the co-culture of human-activated monocytes with NK cells appeared ineffective at first glance. However, unraveling the different factors induced under co-culture conditions revealed that the activating effect of monocyte-released IP-10 on NK cell activity was outcompeted by immunosuppressive PGE2 which was concomitantly produced by the monocytes. Only by adding PGE2 blockers to the system the IP-10 mediated activation of the NK cells was uncovered [10].

The applications described above are used to directly manipulate the functional features of a cell population. Sometimes it is not necessary to interfere with the functional features of cells but to manipulate them just to allow the identification of specific cells. For this purpose, cells of interest can be stained with appropriate labeled antibodies allowing the identification in the suspension. Apart from that, separate cell fractions can also be permanently stained before combining in the co-culture. This can be achieved by using cell staining dyes like carboxyfluorescein succinimidyl ester (CFSE), a fluorescent agent which is cell permeable and covalently binds to intracellular molecules establishing a permanent and stable staining without any so far described side effects. Alternatively, molecular biological methods could be applied such as introducing fluorescent dye genes [9].

9.6 Readouts After Co-culture

9.6.1 Cell Cycle Analysis

Co-culture is not an end in itself. Finally, readouts for the experiment are needed. As important as the effects that can be measured are suitable controls for each setting, for example, using the identical setting of the experiment but leaving the stimulus off. Moreover, controls for the co-culture may also include appropriate monocultures of the involved cellular partners at similar experimental conditions. Possible parameters that could be determined have been mentioned above. Briefly, this could be proliferation. Proliferation means cell division and occurs when cells get stimulated or as a result of tumorigenic transformation

of cells. The cell cycle is divided into different phases G_0; G_1, S, G_2, and M. During the S or replication phase, the DNA is doubled which requires synthesis of new DNA. This step can be used for measuring DNA synthesis by adding a surplus of labeled nucleotides only present in DNA (not in RNA). Thymidine is such a nucleotide. Tagged with a radioactive isotope of hydrogen (^{3}H, tritium), incorporated radioactivity of the DNA can be used as a measure of DNA synthesis directly corresponding to cell proliferation. Alternatively, non-radioactive methods have been established using, for example, fluorochrome-labeled bromodeoxyuridine (BrdU), which is a chemical thymidine analogue that can be incorporated into synthesized DNA instead of thymidine. BrdU incorporation is determined by analyzing the fluorescence intensities of respective cells. More detailed cell cycle analysis can be performed after fixation of the cells in 70% of ice-cold methanol followed by staining with propidium iodide or DAPI. Both of these dyes enter the permeabilized membranes of the methanol-fixed cells and stain the nuclear localized DNA by intercalation. Afterward, these labeled cells can be analyzed by flow cytometry to distinguish the different phases of the cell cycle (G0/G1, S, and G2/M phase). Cell cycle analysis can be performed in separated cell populations after co-culture. If the DNA content of the co-cultured cell types displays significant differences (e.g., when a polyploid tumor cell line is co-cultured with normal diploid MSCs), a cell cycle analysis is even possible directly in the co-culture exhibiting different fluorescence values according to the corresponding cell cycle phases [11].

9.6.2 Analysis of Apoptosis

In contrast to proliferation, cells could also undergo apoptosis as a result of a co-culture. Cells might get apoptotic due to signaling via surface receptors containing death domains after binding to cognate ligands or by soluble factors released by the cellular counterpart (paracrine) or the apoptotic cell itself (autocrine). However, apoptosis could also be induced by strong activation as a physiological regulative mechanism which is called activation-induced apoptosis (AIA). This mechanism normally limits immune reactions in vivo to prevent damage. Apoptosis can, for example, be analyzed by flow cytometry by measuring the surface occurrence of phosphatidylserine as an early event of apoptosis or the intracellular active caspase-3 which is a key enzyme of the apoptotic pathway [12].

Take-Home Message

Each cell culture system requires specific conditions in terms of nutrients, growth factors, temperature, oxygen concentration, pH value, and cell density. Some cells (e.g., tumor cells) can be very easily maintained in culture, while others (e.g., primary cells) might be very fastidious. Once the optimal conditions are defined, most cultured cells can serve for days to weeks as continuous sources for experiments. Co-culture systems, however, are much more complex than just a mixture of two or more established mono cell cultures. General culture conditions of the different cells might be extremely contrary excluding any co-culturing. Growth kinetics of the partners could considerably differ eventually leading to overgrowing of one cell line. In case of immune cells, direct cellular interactions might result in cell stimulation, suppression, or even killing of cell populations. Therefore, optimizing co-culture conditions is indispensable and might take much longer than the initial experiment. Finally, one has to be aware that

genetic and physiological changes of the cells may occur over time probably altering their functional features. Therefore, a careful evaluation of the results obtained from cell culture experiments is mandatory, and comparability with uncultured cells has always to be critically scrutinized.

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Automation of Cell Culture Processes

Jan Hansmann, Ivo Schwedhelm, Andreas Clementi, Dominik Egger, and Cornelia Kasper

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What You Will Learn in This Chapter

This chapter gives insight into commercially available automated cultivation systems for cell culture technologies as well as the theoretical background of process automation and modeling. Exemplary bioreactor systems with sensors, actors, and process principles are described, ranging from the classical stirred tank reactor to more advanced perfused systems. Further, this chapter gives an introduction to principles of process modeling and simulation as well as a theoretical background in process automation and control.

10.1 Bioreactors in the Field of Cell Culture Technology

As discussed in previous chapters, there are different concepts in reactor design. Here we would like to give representative examples of commercially available systems in the field of cell culture technology. One promising candidate is the Mobius® (Merck Millipore) product family. It is a scalable platform of stirred tank bioreactors that provide flexibility by configuring software, hardware, and single-use assemblies for use in suspension and adherent cell culture applications, ranging from 3 to 2000 l in volume. The *Mobius 3 L* bioreactor was introduced in 2009 and thereby standing in the field of stirred single-use bioreactors between laboratory and pilot scale (see ◘ Fig. 10.1).

This unbaffled bioreactor consists of a rigid plastic cultivation vessel with a total volume of 3 l, in which mixing is achieved by a marine impeller. The impeller's diameter is equal to 1/2 the tank diameter offering an off-bottom clearance of 0.028 m. The vessel has a diameter of 0.137 m with an H/D ratio of 1.82, and, for aeration, a microsparger (sintered polyethylene, 15–30 µm pore size) is installed below the impeller [8]. The Mobius 3 L does not have built-in sensors and motor; instead compatibility with various process control

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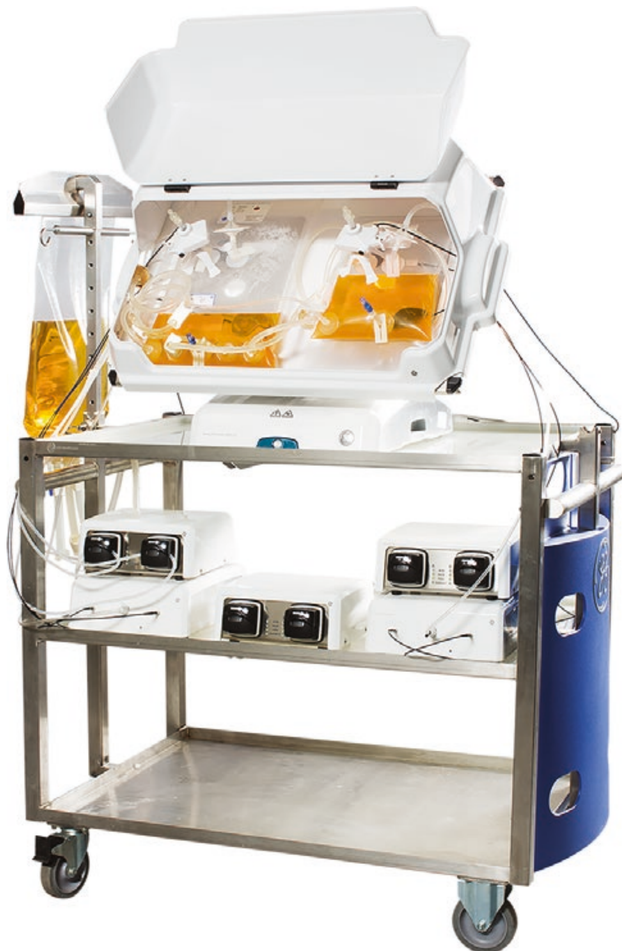
◘ **Fig. 10.1** Mobius 3 L, a disposable stirred tank bioreactor for suspension and adherent cell culture applications. (Image kindly provided by Merck Millipore)

units is granted [3]. For instance, a control unit by ez-Control (Applikon Biotechnology) was already used successfully for measuring pH, DO, and temperature in a CHO suspension culture [5].

For wave-mixed bioreactors, the most widely used and commercially available systems are the WAVE™ bioreactors from GE Healthcare. A first version of the system was first introduced to the market in the 1990s as a disposable alternative to stainless steel bioreactors. It is operated outside of an incubator and consists of a single-use plastic bag filled with culture medium and cell inoculum, placed on a motorized platform. The platform performs a rocking motion, which provides excellent mixing and gas transfer without damaging fluid shear or gas bubbles [12]. Furthermore, the system is equipped with integrated sensor technology and control software to improve automation, reliability, and reproducibility.

Today different versions and scales of the wave-mixed bioreactor family are available to cover the needs of both research and GMP manufacturing applications [14]. The most recent WAVE bioreactor from GE Healthcare is the ReadyToProcess WAVE™ 25 which can be seen in ◘ Fig. 10.2. It is characterized by the 25 liter culture bag, an enhanced rocking motion, and features such as advanced sensors and intelligent control strategies.



◘ **Fig. 10.2** The single-use ReadyToProcess WAVE 25 bioreactor system is a cell culture device for working volumes up to 25 L (Image kindly provided by GE Healthcare)

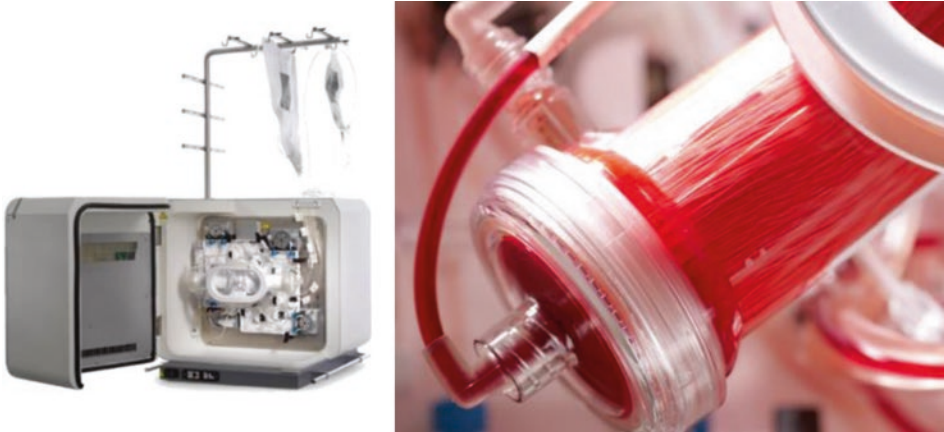


Fig. 10.3 Quantum® Cell Expansion System (Image kindly provided by Terumo BCT)

Commercial hollow fiber systems for adherent cell cultivation are also available, with the Quantum® Cell Expansion System (Terumo BCT) being the most advanced and widely used example. It is a GMP compliant, closed cell culture platform for adherent cells that uses hollow fibers to achieve a high surface-to-volume ratio (Fig. 10.3).

The hollow fiber architecture used with the Quantum system contains about 11.500 single fibers, providing a 2.1 m^2 cell culture surface area, while occupying a total volume of only 180 ml. The same surface area can be achieved with 120 T-175 flasks, but requiring labor-intensive procedures baring risks of contamination. These challenges were resolved with the Quantum system by providing a high degree of automation which allows an adaptation to different cell culture tasks. Via a computer interface peristaltic pumps, fluid sensors and tubing valves are controlled by the user. Additionally, the two-loop system (intraluminal and extraluminal loop) facilitates cell or product harvest. For cell expansion of mesenchymal stem cells (MSC), this system allows to harvest $1.5–3 \times 10^8$ MSCs, but it also needs a high amount ($\sim 2 \times 10^7$) of initial cells and medium [10, 11]. Notably, the Quantum system performs well for clinical applications, but due to the large size and cost, its suitability for the research level is very limited.

10.2 Control of Cell Culture and Tissue Engineering Processes

Basic cellular activities are determined by extrinsic signals sent from the microenvironment in which the cells are embedded. Furthermore, cellular state and functions are influenced by external stimuli such as mechanical, biochemical, or electrical cues [7]. This fundamental mechanism must be considered when culturing cells in a bioreactor. Due to the sensitivity of cells to the provided culture conditions, bioreactor systems comprise control units to ensure reproducible culture conditions that mimic the physiological environment.

A bioreactor system can be interpreted as a dynamic system with in- and output signals (Fig. 10.4). Thereby, the system is represented by the state variables $\underline{x}(t)$ that are influenced by the input variables $\underline{u}(t)$. The output $\underline{y}(t)$ of a dynamic system is a combination of the state variables and can be a physical signal measured by a sensor or a biological

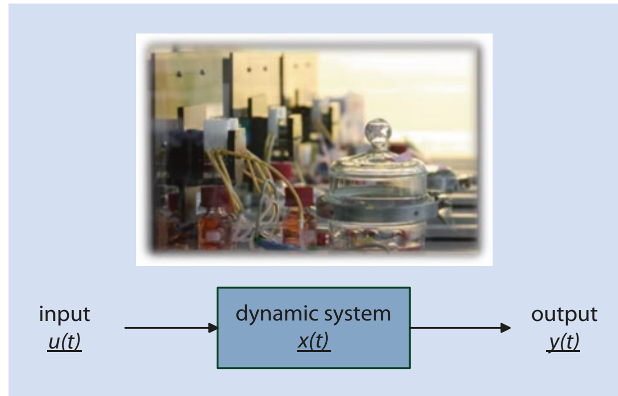


Fig. 10.4 A bioreactor is a dynamic system with input and output signals. The system is represented by the state variables $\underline{x}(t)$ (Image kindly provided by Fraunhofer)

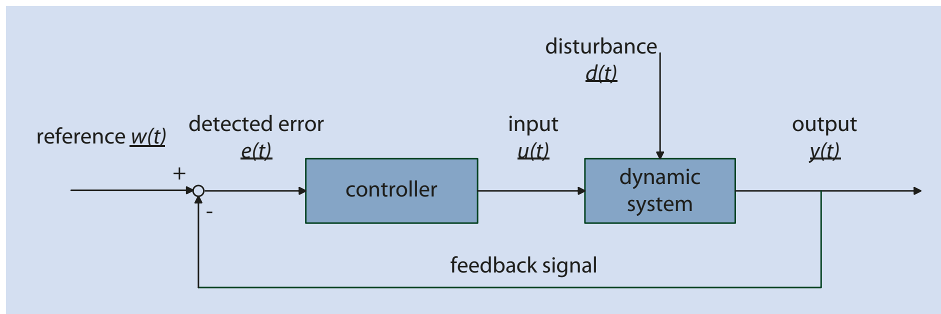


Fig. 10.5 Closed-loop control

parameter such as biomass or metabolite concentrations. In general, the in- and the output as well as the state variables can be scalars or vectors. Due to the relation between input $\underline{u}(t)$ and system state $\underline{x}(t)$, the input signals allow to control a dynamic system.

To ensure optimal process conditions, there are two types of control strategies: open- and closed-loop control. An open-loop controller sets the input of a system independently from the system's output. This mechanism is relatively simple. However, it is only applicable for systems that are robust to external disturbances since deviations from the reference value are not detected and thus not compensated. The renewal of cell culture medium in a bioreactor on a timely basis is an example for an open-loop control application. Without measuring the actual nutrient concentrations, fresh medium restores the nutrients content in the bioreactor to a specific level. Over time, the cultured cells change the composition of the medium by consuming nutrients and releasing waste products. Thus, the medium renewal and the metabolic activity of the cultured cells can induce time-dependent changes of nutrient concentrations. Nevertheless, medium exchange on a timely basis has proven to be suitable for many cell lines and primary cells demonstrating the robustness of this procedure.

In contrast to open-loop control, closed-loop controllers set the input signals depending on the deviation of the system's output from reference value $\underline{w}(t)$ (Fig. 10.5). Therefore, the system's output is monitored, compared to the reference values, and the control action is computed. Thus, this mechanism allows to detect and compensate

external disturbances. An example for a closed-loop control application is the temperature control used in cell culture incubators. A temperature sensor measures the actual temperature in the incubator, and the controller drives a heating system to maintain the required temperature, usually 37 °C. A typical source of disturbance is an increased loss of heat when the incubator door is opened. Moreover, varying ambient temperatures require a controlled introduction of heat. In many cases, stable temperatures in an incubator can be achieved by a simple control mechanism, which is called two-point controller. When the measured temperature drops below a specific threshold, which is equal or lower than the required reference temperature, the heating system is activated. However, introduced heat is usually not controlled and depends on the heating power and the characteristics of the heated space. If the temperature rises above a second temperature threshold, which is higher than the first threshold value, the heating system is switched off. During operation, the temperature oscillates between the two threshold temperatures in the range of the required reference value.

A very frequently used controller that allows more consistent characteristics of the control value is the proportional-integral-derivative controller (PID controller) (Fig. 10.6). For a scalar system, a PID controller applies a correction to the system's input $u(t)$ based on the continuously calculated error $e(t)$ between the reference value and the measured system output. Thereby, the correction is the weighted sum of a proportional, an integral, and a derivative term of $e(t)$. The controller can be adjusted to the given system by the parameters k_P, k_I, and k_D. While the proportional term accounts for the actual error, the derivative term accounts for the current rate change and thus for a predicted trend of the error. As the integral term accumulates $e(t)$, even small values can induce a strong response over time. The integral term can also prevent a permanent control deviation [6]. If the controller works properly, it minimizes $e(t)$ over time and can even change unstable system dynamics into a stable behavior.

PID controllers do not necessarily require knowledge on the underlying process and are thus applicable for many tasks in different fields like engineering, biotechnology, or automotive. To adapt the controller on a specific process or to ensure robust control characteristics, heuristic tuning methods are described [13]. Therefore, defined input signals such as step functions are applied to the non-controlled system, and k_P, k_I, and k_D are calculated from the response. For some systems, such as power plants or sensitive communication systems, applying a test function to the input is not possible due to safety or economic reasons. Here, a controller must be designed based on information on the respective process. To gain mechanistic understanding of the process to be controlled, computational modeling can be applied.

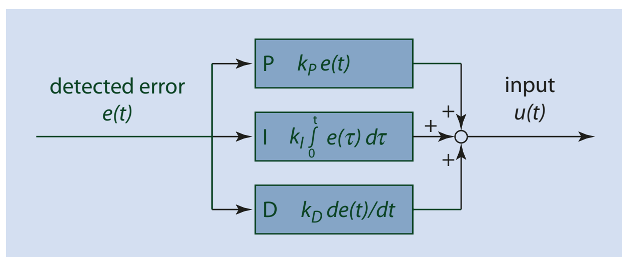


Fig. 10.6 Proportional-integral-derivative controller

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10.3 Mathematical Modeling and Simulations

Computer simulations facilitate to study complex systems by deriving functions that describe the system's dynamics. A transducer to measure the pressure in a bioreactor is an example for a modeling application. For simplification, it can be regarded as a spring and mass system (Fig. 10.7). In a pressure transducer, a small mass is moved from the resting position in dependency of the pressure F_P (N) that is monitored. A spring and a damper, both attached to the mass and the housing of the transducer, generate counterforces bringing the mass back to the resting position when the external force disappears. Therefore, the spring force F_C (N) acts in the opposite direction to the motion denoted by the position $x(t)$. In addition to the spring, the damper dissipates energy from the system. The force F_D (N) induced by the damper reacts in the opposite direction to the velocity $dx(t)/dt$. The pressure transducer can be interpreted as a dynamic system, whereby the system's input is represented by the force F_P and the system's output is the resulting position $x(t)$ of the mass m. In a real transducer, this position is subsequently converted into an electrical signal. The dynamics of the transducer, and thus the signal transmission, is determined by the mass m (kg), the spring constant C (N/m), and the damper constant D (Ns/m).

Applying Newton's law, a differential equation revealing the connection between the system parameters and the system dynamics can be derived.

$$\sum F = m\frac{d^2x(t)}{dt^2} = F_C + F_D + F_P$$

$$m\frac{d^2x(t)}{dt^2} = -Cx(t) - D\frac{dx(t)}{dt} + F_P$$

Thereby, $md^2x(t)/dt^2$, $Cx(t)$, and $Ddx(t)/dt$ represent the transducer dynamics, whereas F_P denotes the external input. Depending on the systems parameter m, C, and D, the obtained ordinary differential equation of second order can exhibit a free oscillatory behavior or oscillatory characteristics with an amplitude gradually decreasing to zero. Moreover, when exceeding a critical damping, also an asymptotic behavior is possible [2].

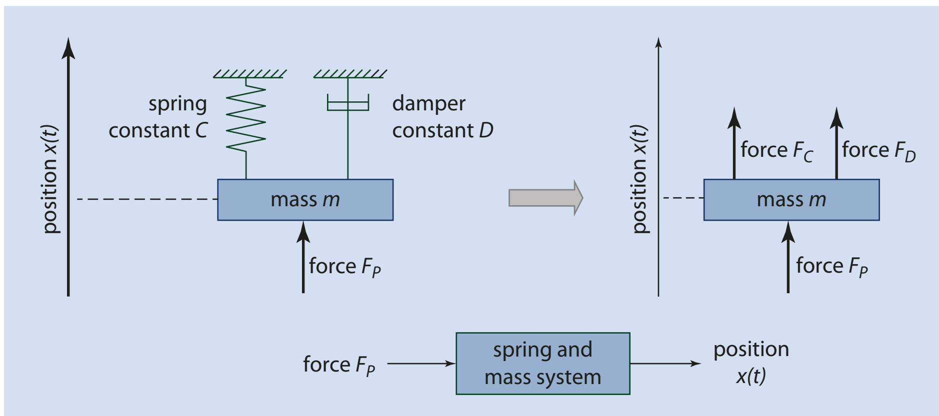


Fig. 10.7 A spring and mass system in a pressure transducer

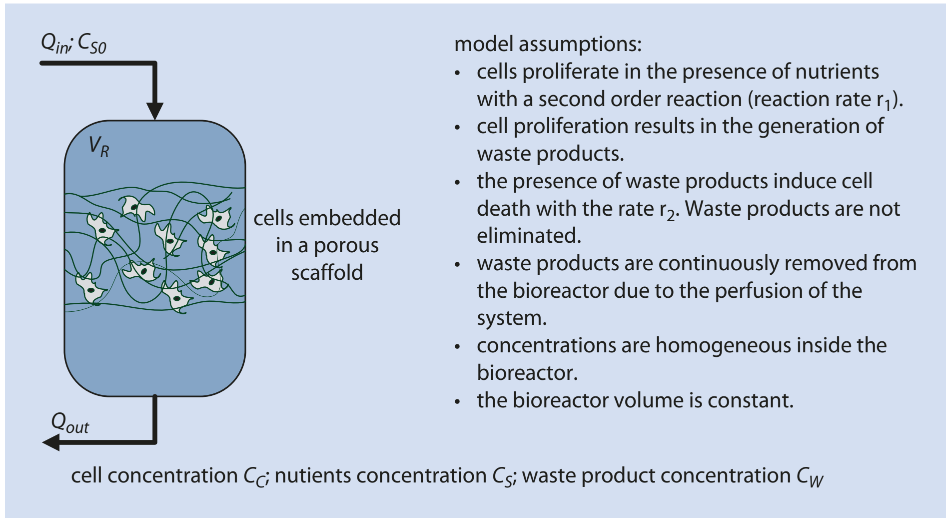


Fig. 10.8 Model for cells cultured in a perfusion bioreactor

In addition to physical systems such as the described pressure transducer, also biological aspects of cell culture processes can be addressed by mathematical modeling and simulation. An example is the growth of adherent cells in a perfused bioreactor (Fig. 10.8).

In this example, cells are embedded in a porous scaffold avoiding that cells are removed from the culture volume V_R (m^3). Over time, cells proliferate depending on the nutrients C_S (g/m^3) as well as the cell concentration C_C (g/m^3), respectively. The proliferation results in an increase of biomass C_C and additionally in the production of waste products C_W (g/m^3). In the given example, the presence of waste products entails a decrease of the cell concentration. Cell proliferation and cell death are characterized by the reaction rates r_1 ($m^3/g/s$) and r_2 ($m^3/g/s$). To prevent high waste product concentrations and to provide a sufficient amount of nutrients, the bioreactor system is perfused with cell culture medium with a nutrients concentration C_{S0} (g/m^3) at the bioreactor inlet. Hereby, Q_{in} (m^3/s) denotes the inlet flow rate. Outflowing medium with a flow rate of Q_{out} (m^3/s) at the bioreactor outlet contains both soluble components, nutrients and waste products in the respective concentrations. For the model, C_C, C_S, and C_W represent the state variables of the dynamic system. Based on the model assumptions and the reaction rates, the bioreactor can be described by a system of differential equations:

$$\frac{dC_C}{dt} = r_1 C_S C_C - r_2 C_C C_W \tag{10.1}$$

$$\frac{dC_S}{dt} = \frac{Q_{in}}{V_R} C_{S0} - r_1 C_S C_C - \frac{Q_{out}}{V_R} C_s \tag{10.2}$$

$$\frac{dC_W}{dt} = r_1 C_S C_C - \frac{Q_{out}}{V_R} C_W \tag{10.3}$$

The first differential equation represents the change of the biomass and considers cell growth and cell death with the respective rates r_1 and r_2. Hereby, cell growth induces a

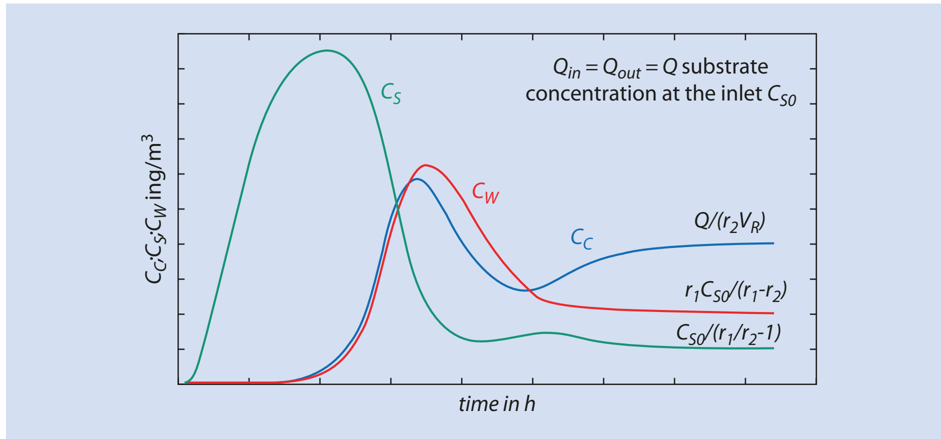


Fig. 10.9 Qualitative time course of the cell concentration C_C, the nutrients concentration C_S, and the waste product concentration C_W

positive change indicated by the positive sign, whereas cell death decreased the biomass concentration, indicated by a negative sign. To note, only if both species, cells and nutrients, or cells and waste products are present, the two products are greater than zero and contribute to the concentration change. The second differential equation describes the change of the nutrients. In addition to the consumption of nutrients for cell growths, nutrients are introduced or removed by inlet or outlet volume flow. Finally, the third differential equation accounts for the production of waste products and the removal thereof due to the bioreactor perfusion.

By using a numerical solver, the solution of the differential equation system can be computed following the definition of initial values for the three species. When assuming that at the beginning of the culture process only a small amount of biomass and no nutrients as well as no waste products are present in the bioreactor, a typical time course for C_C, C_S, and C_W is depicted in Fig. 10.9. Initially, the nutrient concentration increases quickly, whereas the cells remain in a lag phase. Following this lag phase, the cell concentration rises rapidly and entails a strong increase of waste products. The high proliferation of cells consumes high amounts of nutrients. Due to the waste products, the cell concentration reaches a maximum and decreases gradually to a steady-state level.

The initial transient behavior depicts the dynamic of the process; however for real-life applications, the steady state is of higher interest. The steady-state solution reveals the productivity of the bioreactor run at the given parameters. For many processes, the steady-state solution can be calculated analytically by setting the changes of the concentration to zero:

$$0 = r_1 C_S C_C - r_2 C_C C_W \tag{10.4}$$

$$0 = \frac{Q_{in}}{V_R} C_{S0} - r_1 C_S C_C - \frac{Q_{out}}{V_R} C_s \tag{10.5}$$

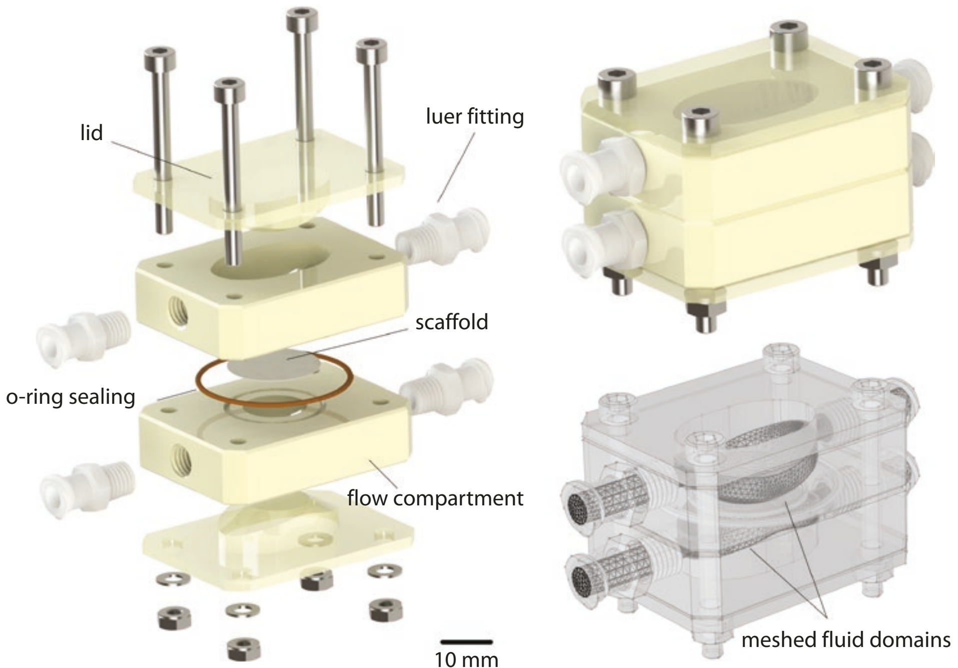
$$0 = r_1 C_S C_C - \frac{Q_{out}}{V_R} C_W \tag{10.6}$$

Thereby, the system of differential equations is converted into a system of nonlinear equations. The solutions for C_C, C_S, and C_W are shown in ◘ Fig. 10.9. Interestingly, the analytical solution of the system for steady-state conditions clearly reveals the impact of the technical parameters such as the reactor volume V_R or the perfusion rate Q on the final cell concentration, which allows the optimization of the investigated cell culture process. Although the described mathematical model facilitates mechanistic understanding, it renders a simplification of the real cell culture experiment. For example, concentrations in the bioreactor were assumed as homogeneous and the fluid dynamics were neglected. To study such aspects, sophisticated finite element method (FEM) is available.

10.4 Finite Element Method

The FEM is based on numerical procedures to approximate the solution of complex processes that are represented by partial differential equations [4]. The principle of the FEM is to divide a mathematical model into a number of smaller parts – the finite elements – which are easier to handle compared to the initial model. To solve the mathematical problem, all finite elements are converted into a system of equations. In its practical mode of operation, the FEM includes the generation of a mesh, whereby the mesh elements are associated to small spatial areas in the system of interest.

An example for a FEM application is the characterization of the fluid dynamics inside a bioreactor that is used to culture cells on a scaffold under shear stress conditions (◘ Fig. 10.10). The system comprises several in- and outlet ports for the perfusion with the medium. The scaffold or tissue is mounted in the middle of the bioreactor and thereby separates two



◘ **Fig. 10.10** Computer-aided design of a flow bioreactor. Left, explosion view; top right, collapsed view; bottom right, FEM mesh applied to the reactor geometry

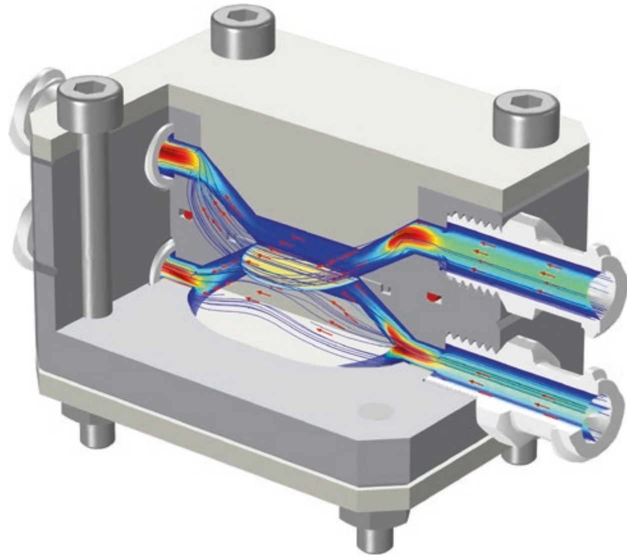


Fig. 10.11 FEM-based simulation on the fluid velocity field inside the reactor during operation

fluid compartments. While in operation, cells on the scaffold are exposed to pressure and mechanical shear stress that dictate tissue development [9]. However, due to the complex geometry of the bioreactor, the correlation between flow conditions and mechanical stress on the scaffold is difficult. Nevertheless, this problem can be investigated by FEM. In a first step, the geometry data of the bioreactor is harnessed to create a three-dimensional mesh that subdivides the domain of interest into a finite number of elements (Fig. 10.10).

Thereby, the fluid inside the bioreactor is the relevant domain for the prediction of the mechanical culture conditions, whereas the solid bioreactor components represent boundary domains. Following the definition of the model geometry, the underlying physics must be applied to the respective domains. In this case study, the Navier-Stokes equation solving for laminar fluid flow is applied to the fluid domain, representing the traveling cell culture medium. For the simulation, also model parameters such as material properties like the fluid density or viscosity are required in addition to the temperature or pressure. The definition of boundary and initial conditions finally allows to derive the solution (Fig. 10.11). In general, the solution can be a steady-state solution or a time-dependent transient solution that usually entails higher computational costs. The computation of the steady-state solution is based on a system of equations, while the transient solution is characterized by a system of ordinary differential equations [1].

The FEM is also a powerful tool to optimize the design of a bioreactor system by evaluating different reactor geometries *in silico* prior to manufacturing. In contrast to experimental design optimization, the FEM-based approach is more cost- and time-efficient and provides information on physical problems that are hardly accessible by experimental approaches. For example, Fig. 10.12 depicts the flow dynamics of three different bioreactor designs. The first design lacks a homogeneous shear stress distribution across the cell-seeded scaffold (red arrow). By increasing the width of the flow chamber, shear stress heterogeneity can be reduced. However, turbulences at the inlet region (yellow arrow) as visible for the second design indicate undefined culture conditions, e.g., when introducing a cell suspension during seeding. The third design is optimal regarding shear stress distribution on the scaffold surface and alignment of the streamlines. Therefore, the incoming medium is guided through the bioreactor by a very smooth flow compartment with no sharp edges.

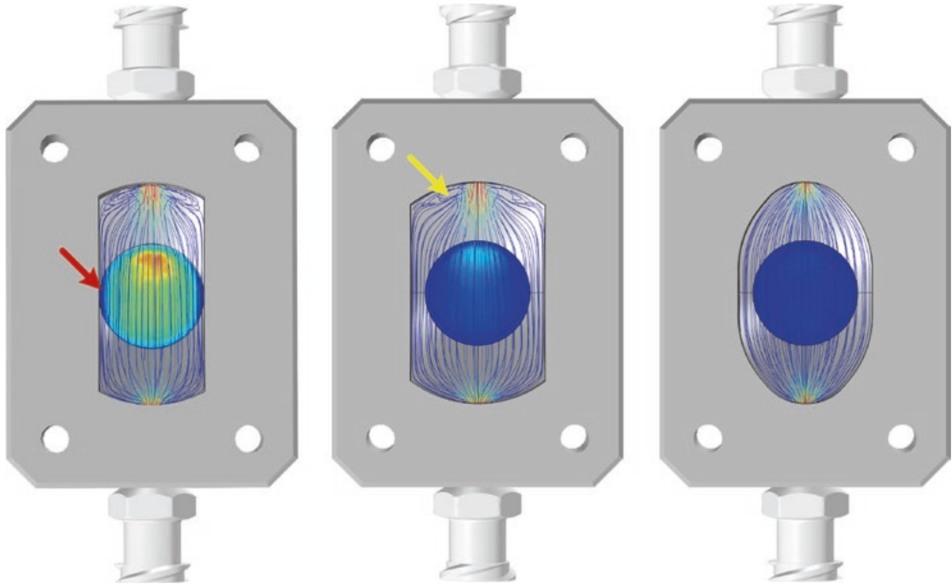


Fig. 10.12 Fluid flow simulations on a variety of fluid bed designs

Besides the general optimization of the bioreactor's flow chamber, FEM computations are also a powerful predictive tool when systematically assessing mechanical parameters. For instance, the physical shear stress that primarily occurs on the scaffold surface is dictated by the height of the flow chamber above the scaffold membrane. Here, it is of main interest to characterize the magnitude of shear stress depending on the flow chamber geometry. To investigate this dependency, most FEM software tools offer the possibility to perform a sequential parametric sweep of one or more parameters of interest. In this case, the channel height is defined as geometric design parameter h (mm), and its value is altered throughout a set of otherwise unchanged subsequent flow simulations. The resulting shear stress pattern for a channel height of 1 mm, 2 mm, and 3 mm, respectively, is indicated by objective color code in Fig. 10.13. Naturally, the parameter value resolution can be adjusted to the required amount. However, a higher resolution of the parameter leads to a proportional increase of simulations and is thus a time-consuming effort. It is therefore necessary to carefully consider the parameter sweep interval in order to achieve a good and time-efficient data readout.

The obvious strength of parametric sweeps lies in its unlimited flexibility of test values. In this example, only the channel height is defined as sweep parameter. However, additional parameters, e.g., inlet flow velocity, fluid temperature, and system pressure, can be defined, and every parameter combination will result in a separate solution of the fluid flow regime. Due to the convenient testing of multiparameter combinations, parametric sweeps are highly useful during designing and optimizing bioreactor vessels.

The result of a parametric sweep study is a readout factor in dependency of the respective design parameter (Fig. 10.14). The solution dimension is determined by the number of investigated parameters.

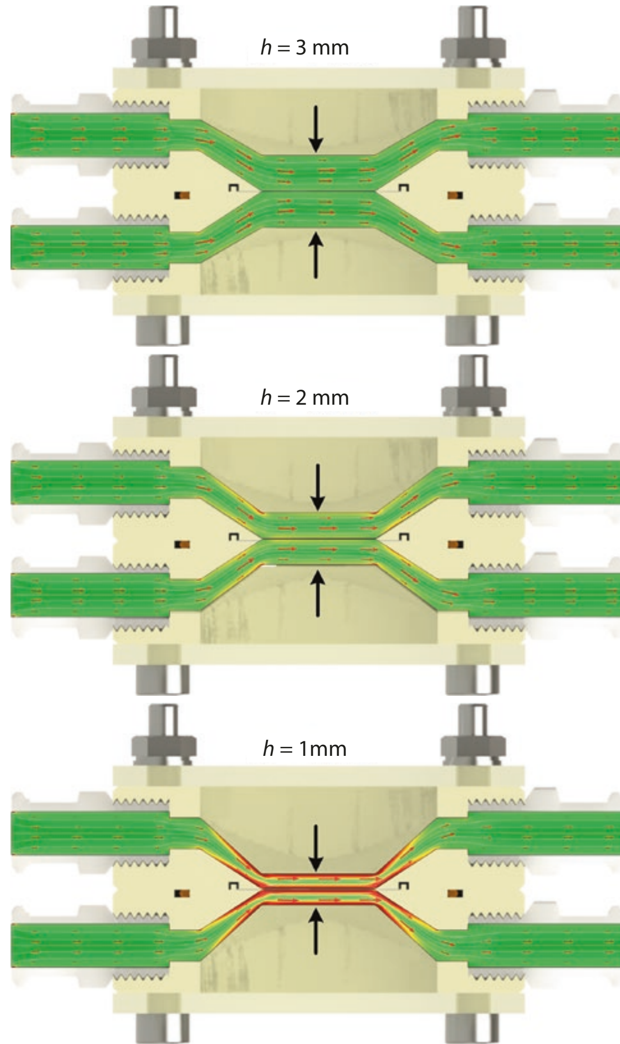


Fig. 10.13 Parametric sweep on flow channel height h and its predictions on occurring shear stress

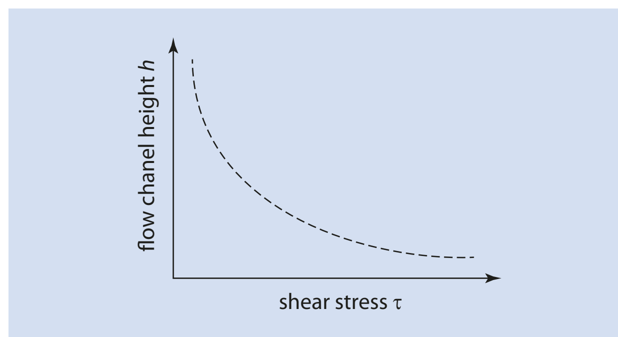


Fig. 10.14 Exemplary interpolation as a result of channel height h parametric sweep on shear stress τ inside the flow reactor at constant flow rate

Take-Home Message

Although cell culture and tissue engineering have a strong biological and medicinal focus, methods from engineering sciences have been proven valuable. The mathematical interpretation, e.g. by analytical or numerical modeling, offers answers to complex biological questions. Even advanced cultivation systems can be broken down to single components, which play their part in one or more control loops. Understanding the technical as well as the biological dynamics is crucial to the automation of bioprocesses.

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