Drug Testing In Vitro

Breakthroughs and Trends in Cell Culture Technology

Edited by
Uwe Marx and Volker Sandig

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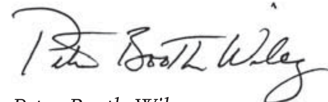

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Foreword

In recent years, few methods have changed so dramatically as those used *in vitro* for drug development. The main areas of progress involve target finding and validation with molecular biology, and *in-vitro* testing of drug safety to the production of biological molecules. The application of these methods has made all steps of drug development not only faster, but also less costly.

As research in all areas is continuing apace with major efforts, we can expect major breakthroughs with the maturation of human micro-organoid *in-vitro* cultures in the near future. Clearly, however, because of the increased sophistication and specialization of these investigations, an even greater need for team work is indicated.

Hence, it is mandatory for those of us involved in drug development to keep pace with the continuous progress in methodology, by receiving the experts' overview, as presented in this book.

Frankfurt a. M., July 2006

Prof. Dr. Rolf Krebs
former Chairman of the
Board of Managing Directors
of Boehringer Ingelheim GmbH

Preface

At the beginning of the 21st century, the development of medicine is suffering from two major obstacles.

First, new drug candidates directed at *pivotal human receptors* can have unprecedented positive or negative biological effects involving systemic interactive networks specific to humans. None of the animal species or human cell lines can properly imitate the biological effects on these networks. Consequently, few relevant data on the efficacy and safety of new drugs can be obtained for evaluation prior to human testing. A prime example is the super-agonist antibody TGN1412, which was developed to direct the immune system to fight cancer cells or to reduce arthritis pain, and has triggered multiple organ failure in healthy volunteers undergoing experimental testing. In binding the CD28-receptor, the antibody overrides the basic control mechanism of the whole immune system. Yet whilst adhering to standard clinical research guidelines, the drug showed absolutely no adverse effects in studies with animals.

Second, significant drawbacks – such as severe adverse side effects – often occur after drugs have entered the market. Today, there are increasing indications that *specific genetic predisposition* is one of the key reasons for these high-profile recalls. This human genetic diversity is rarely addressed in preclinical and clinical safety studies at the present time. A sound hypothesis on the correlation of morbidity of patients treated with roferoxib (Vioxx) with the genotype for 5-LOX and 5-LOX activating protein polymorphisms, is one of many examples describing this obstacle.

The breakthrough might be to develop high-throughput, human micro-organoid *in-vitro* test systems. In mammals, organs and systems are built up by multiple identical functionally self-reliant structural units, with easily remembered examples *in vivo* being the liver acinus, β-cell islets in the pancreas, alveoli in the lung, or germinal centers in lymph nodes. When science and industry succeed in designing human micro-organoids *in vitro* that fully emulate these *in-vivo* counterparts, the dream of drug testing predictive to individual human exposure might became reality. For at least 30 years the vision of proper modeling of these human micro-organoids *in vitro* to gain knowledge about their performance and function in man – and consequently to use them for highly predictive drug screening and testing purposes – has been set back by prohibitive scientific and technological bottlenecks. However, achievements made over the past seven

years have substantially changed this starting position, and the multidisciplinary contributions in this book introduce different aspects leading towards anticipated short-term progress in that area.

- Part I brings together an overview of new and forthcoming tissue models, the challenges to be met by the development of bioreactors, and the biosensoric microstructures for control and measurement. An illustration of complexity is provided by the biomonitoring of airborne contaminants *in vitro*.
- Part II combines overviews of state-of-the-art *in-vitro* techniques in conventional monolayer and suspension culture systems, with the potential of two relatively new technological platforms – the creation of human designer cell lines and stem cell technologies. The latter provides basic guidelines of how to overcome the chronic bottleneck of sustainable, human genotyped cell and tissue supply.
- Part III emphasizes the tension between ethical, regulatory and commercial aspects of drug testing and screening on human micro-organoids *in vitro* as a viable alternative to animal testing.
- Part IV concludes with the tremendous potential of the anticipated emerging *in-vitro* drug evaluation platform technology, including a road map enforcing them.

The book is introduced by a personal statement of Rolf Krebs, former chairman of the Board of Managing Directors of Boehringer Ingelheim.

Progress anticipated in the emerging platform technology can have significant impact beyond the borders of drug screening and testing. In Europe, at least, legislative pressures such as the cosmetics directive and the retrospective REACH (Registration, Evaluation and Authorisation of Chemicals) program for 30 000 chemicals, has created a dramatic increase in industrial interest in predictive human *in-vitro* tissue culture test systems for the evaluation of cosmetics, chemicals, or nutraceuticals. Hence, this book also provides useful inside information for professionals from those areas.

Finally, the book would not exist with the outstanding creative assistance of Silke Hoffmann and Philip Saunders.

Berlin, October 2006 — *Uwe Marx and Volker Sandig*

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Part I
Emerging *In-Vitro* Culture Technologies

1
Intelligent Biomatrices and Engineered Tissue Constructs: *In-Vitro* Models for Drug Discovery and Toxicity Testing

*Philip Lazarovici, Mengyan Li, Anat Perets, Mark J. Mondrinos,
Shimon Lecht, Christopher D. Koharski, Paul R. Bidez III,
Christine M. Finck, and Peter I. Lelkes*

1.1
Introduction

The rapid progress in combinatorial chemistry continues to yield a myriad of potentially bioactive compounds every day. With thousands of pharmaceutically valuable drugs at hand, there is an urgent need for engineered tissue equivalents that could serve as *in-vitro* model systems during the initial stages of drug discovery, specifically during the preclinical stages of cell/tissue-based high-throughput screening (HTS). Given the well-known problematics of using two-dimensional (2D) cell cultures as pharmacological test-beds, more realistic three-dimensional (3D) tissue constructs are required. Generation of high-fidelity engineered tissue-like constructs is based on the targeted interactions of organ-specific cells and "intelligent" biomimetic scaffolds, emulating the complex natural environment, the extracellular matrix (ECM) in which these cells develop/differentiate and function.

In this chapter, we will begin by introducing the most common natural and synthetic materials and platform biotechnologies for creating scaffolds that are in use for engineering tissue constructs, and which might be useful for pharmaceutical purposes, as models for drug discovery and *in-vitro* testing. A variety of approaches for modifying the chemistry and geometry of these materials towards rendering them useful as intelligent biomatrices for 3D tissue engineering will be presented. We then will discuss, both paradigmatically and critically, the fabrication principles and potential use of engineered constructs in cardiac, hepatic, and pulmonary tissue engineering. Finally, we will explore in more detail the current and future use of engineered models of the blood–brain barrier (BBB), where the use of novel "intelligent" biomaterials and scaffolds promises to break the current impasse in engineering high-fidelity *in-vitro* models of the BBB and thus facilitate reliable and predictive HTS of central nervous system (CNS)-active compounds during early stages of drug discovery.

Although the application of bioengineered 3D *in-vitro* tissue models in pharmaceutical research is in its infancy, the interdisciplinary approach and the achievements described in this chapter provide an encouraging first step towards the accelerated development of such models for drug discovery.

1.2 Intelligent Biomaterials and Scaffolds for Tissue Engineering

Currently, a broad range of synthetic polymers are being used for scaffolding, including poly(ε-caprolactone) (PCL), poly(L-lactide-*co*-ε-caprolactone) (P(LLA-CL)), polyglycolic acid (PGA), polylactic acid (PLA), or copolymer poly (lactidic-*co*-glycolic) (PLGA) [1–4], as well as natural biomaterials such as alginate, elastin, collagen, or gelatin [5–9]. Ideally, all scaffold materials should be nontoxic, biocompatible, biodegradable, and nonimmunogenic. Moreover, for use *in vivo*, scaffolds should – with only few exceptions such as cartilage and cornea – be able to induce angiogenesis to facilitate blood supply to, and waste removal from, the newly formed tissues. The "intelligence" of a biomaterial and the ensuing scaffolds can be gauged from its competence to induce and maintain organ-specific differentiation and function of the cells growing in/on them and generating tissue-like constructs.

1.2.1 Synthetic Materials

One of the main advantages of synthetic materials is the ability to precisely control their physico-chemical properties, such as molecular weight of the polymer, strength, degradation time, mechanical properties, and hydrophobicity [10]. Amongst the most widely used polymers in tissue engineering are the poly (α-hydroxy acids) of aliphatic polyesters, such as PLA, PGA, and PLGA [11]. These synthetic polymers can be produced in numerous physical forms, including meshes, sponges, and films, and molded into many shapes, depending on the type of tissue one wishes to emulate (e.g., heart, kidney, ear). The rate of biodegradation of PLGA scaffolds depends not only on the ratio of lactide and glycolide, but also on whether the polymer is mixed with polycaprolactone, collagen, or other synthetic polymers [12–14]. Cell attachment can be improved by covalently modifying the polymers, or by passively coating the scaffolds [15]. Growth factors can also be incorporated into the matrix in order to improve biocompatibility [16].

Since their discovery some 30 years ago, electrically conductive polymers – also known as "synthetic metals" – have been used in many areas of applied chemistry and physics, such as light-emitting diodes and batteries [17]. More recently, there has also been a growing interest in conductive polymers for diverse biomedical applications, specifically as conductive scaffolds for cardiac and neural tissue engineering. The rationale for using conductive polymers is based on the fact that the eukaryotic cell plasma membrane is charged and that, specifically in neurons

and myocytes, a multitude of cell functions, such as attachment, proliferation, migration and differentiation could be modulated through electrical stimulation [18–22]. Common classes of organic conductive polymers include polyacetylene, polypyrrole (PPy), polythiophene, polyaniline (PANi), and poly(para-phenylene vinylene). Some of these conductive polymers (especially PPy) have found certain biomedical applications, such as for the immobilization of proteins [23, 24]. Christine Schmidt and her co-workers were the first to employ PPy for tissue engineering purposes [18, 25–28]. Interestingly, this group most recently described a novel 12-mer peptide (T59) that selective binds to conductive PPy and promotes cell attachment [29] This peptide may become useful for immobilizing a variety of bioactive molecules on PPy and other synthetic/conductive polymers, without altering their bulk properties.

Some recent studies have used PANi, another well-characterized organic conducting polymer, as an electroactive substrate for tissue engineering applications [30–33]. PANi is biocompatible *in vitro* and in long-term animal studies in *vivo* [31]. To date, most of these studies have investigated the biological properties of PANi solvent-cast into 2D films, rather than engineered into 3D (nano) fibrous scaffolds. A few years ago, Díaz et al. [34] reported that doped, conductive PANi blended with polystyrene (PS) and/or polyethylene oxide (PEO) could be electrospun into nanofibers. In extending these studies, we recently co-electrospun PANi with gelatin, for instance denatured collagen, to yield nanofibrous scaffolds which are both highly biocompatible and electroactive, and may be suitable for applications in cardiac and cardiovascular tissue engineering [35].

1.2.2 Natural Biomaterials

Natural biomaterials for scaffold fabrication include both purified ECM proteins, such as collagen, elastin, ECM derivatives, such as Matrigel™ and small intestinal submucosa (SIS™) acellular matrix, as well as materials derived from marine plants and crustaceans, such as alginate and chitosan. Natural biomaterials more closely mimic, than synthetic polymers, both function and structure of the native extracellular environment. Natural biomaterials, such as collagens, are largely conserved among different species and provide a readily available source of materials for tissue engineering. Importantly, when used as 3D matrices – either as hydrogels or as fibrous or porous scaffolds – these materials can serve as ubiquitous (but occasionally also species-or tissue-specific) templates for cell attachment, growth and differentiation.

Matrigel™, an ECM derivative isolated from the murine Engelbreth-Holm-Swarm (EHS) sarcoma, is a complex mixture of basement membrane proteins, mostly laminin and type IV collagen, which also contains a large number of essential growth factors and cytokines. Unlike artificial synthetic scaffolds, Matrigel™ provides a natural, biocompatible environment [36], which induces organotypic differentiation of cells cultured on or in this hydrogel, because of the complexity of its composition and its viscoelastic properties. Given its biological

complexity, Matrigel™ provides an excellent differentiative environment for *in-vitro* tissue engineering application; its use *in vivo* in animal models is mainly restricted to syngeneic mice. Sheets made of the acellularized porcine SIS are in clinical use as well-tolerated, xenogeneic scaffolds, inducing variable degrees of tissue-specific remodeling in the organ or tissue into which it is placed [37]. SIS is mostly composed of type I collagen, though it has some type III and type IV collagen in addition to other ECM molecules, such as fibronectin, hyaluronic acid, chondroitin sulfate A and B, heparin, and heparin sulfate, and some growth factors, such as basic fibroblast growth factor (bFGF), transforming growth factor (TGF), and vascular endothelial growth factor (VEGF) [37].

Collagen and elastin are two key structural ECM components in many tissues [8, 13]. These proteins are important modulators of the physical properties of many types of engineered scaffolds, affecting cellular attachment, growth and responses to mechanical stimuli [38, 39]. Matthews et al. [40] and Boland et al. [41] were the first to generate 3D micro- and nano-fibrous scaffolds from collagen and elastin for cardiovascular tissue engineering by electrospinning (see below). Tropoelastin, the cellular precursor of elastin, is secreted from elastogenic cells as a 60-kDa monomer that is subjected to oxidation by lysyl oxidase. Subsequent protein–protein associations give rise to massive macroarrays of elastin, for example, in the inner elastic lamina of arterial blood vessels. As a consequence, elastin is a substantially insoluble protein network that displays elasticity, resilience, and biological persistence. Soluble elastin is typically available either as fragmented elastin in the form of alpha- and kappa-elastin [42], or through expression of the natural monomer, tropoelastin [43]. Recently, tropoelastin was also electrospun into scaffolds for tissue engineering purposes [9].

Alginate hydrogels (generally 1%, w/v in water) can change their physical state towards hydrogel, depending on the cross-linker and calcium chloride concentration [44]. Due to their versatile viscoelastic properties and adjustable porosity (> 80%), alginate scaffolds have been used for a number of diverse tissue engineering applications such as the liver [45] or pancreas. Alginate scaffolds could also provide a charged surface environment to facilitate the 3D culturing of cardiac cells, and can also be used for regeneration and healing of the myocardium after heart failure [46].

The complexity of the ECM composition *in situ* makes it difficult to fully emulate the "organ-specific environment" *ex vivo*, either by design and/or synthesis. However, the use of natural biomaterials, either alone or in combination with other natural or synthetic polymers, such as collagen/glycosaminoglycans or collagen/PLGA, may improve the biocompatibility of the ensuing scaffolds by reducing inflammatory responses *in vivo* and improving initial cell attachment and differentiation. Cells growing in such an instructive environment are stimulated to remodel this "provisional matrix" in a tissue-specific fashion; thus, these "naturally intelligent matrices" provide the necessary cues for organotypic differentiation and assembly of engineered tissue constructs.

1.3
Fabrication of Scaffolds for Tissue Engineering

As discussed above, an ideal scaffold for tissue engineering must provide the necessary mechanical/structural support and contain the appropriate instructive/differentiative cues, such as the capability of inducing neovascularization, to allow the tissue-engineered construct to be integrated into the host surroundings [47]. Most scaffolds presently being investigated in animal research promote some cellular ingrowth, and may hence be quite useful for *in-vitro* applications. Overall, however, most of these scaffolds pose significant limitations to host integration *in situ*, including a host-versus-graft immunological response, and do not offer a very effective basis for organ replacement. Therefore, there is a need to develop novel scaffolds and approaches.

In addition to using hydrogel-based scaffolds made of collagen or fibrin, several novel techniques have been developed for engineering 3D "solid" scaffolds with enhanced mechanical properties. The microscopic/nanoscale structure and function of biological macromolecules constituting conventional hydrogels are important for cell physiology. However, the relatively weak mechanical properties of hydrogel scaffolds pose a major drawback, especially *in vivo*. Thus, diverse "solid" scaffolds, made of water-soluble polymers (collagen, fibrin, and alginate) with improved mechanical properties have been engineered using controlled freezing and thawing procedures, followed by crosslinking. Such scaffolds have shown excellent biocompatibility and facilitated excellent cellular ingrowth both *in vitro* and *in vivo*. More recently, solid nano/microfibrous scaffolds have been generated by electrospinning or acellularization (see Section 1.3.1). These fibrous scaffolds more realistically emulate salient structural and biological features of the natural ECM, and seem to be very well suited as substrates for 3D tissue engineering purposes, both in *in vitro* and *in vivo*. One of advantages of 3D nanofibrous scaffolds is the small diameter of the fibers, which is similar to the diameters of ECM proteins *in situ*. Such small fiber diameters provide a relatively large surface-to-volume ratio, enabling the absorption of liquids and facilitating cellular attachment and cell–cell interaction. These scaffolds also exhibit unique mechanical properties which permit better cell penetration and proliferation within the scaffolds as compared to 3D hydrogels. Recent data have suggested the possibility of generating hybrid scaffolds for cardiac tissue engineering by combining hydrogel and solid scaffolds comprised of both synthetic and natural biopolymers such as PLGA, collagen, or elastin [13].

1.3.1
Electrospinning

The process of electrospinning, which has been well known for many years in the textile industry and in organic polymer science [48–50], has recently emerged as a novel tool for generating biopolymer scaffolding for tissue engineering [51]. This is a process for the production of polymer filaments using an electrostatic force

[52, 53]. In this process, a polymer solution is introduced into the electrical field generated by a high-voltage power supply. The polymer filaments are formed from the solution traveling between two electrodes bearing electrical charges of opposite polarity. Upon ejection from a metal spinneret through a small hole, the solvent in the charged solution jet rapidly evaporates, thus generating ultrathin fibers, which then are deposited onto the collector [54]. Optimization of this technique depends to a large part on the material to be electrospun, and involves adjusting crucial parameters, such as the nature of the solvent and the concentration of the solute, as well as the potential difference and the distance between the electrodes [9]. Electrospinning is a novel, increasingly important platform technology for producing nanofibrous scaffolds from a variety of polymer materials, including synthetic polymers, natural proteins, and blends of natural and synthetic materials [9, 35, 55]. Figure 1.1 illustrates typical (autofluorescent) light-microscopic images of electrospun elastin (Fig. 1.1A) and gelatin (Fig. 1.1B), as well as scanning electron microscopy (SEM) images of PLGA (1.1C), and a blend of PANi-gelatin fibers (Fig. 1.1D).

The topology of these electrospun scaffolds closely mimics that of the native ECM; it is particularly striking in the case of the wavy appearance of elastin, reminiscent of the elastic lamina in blood vessels. Depending on the spinning conditions, fibers with diameters in the range from several micrometers to less

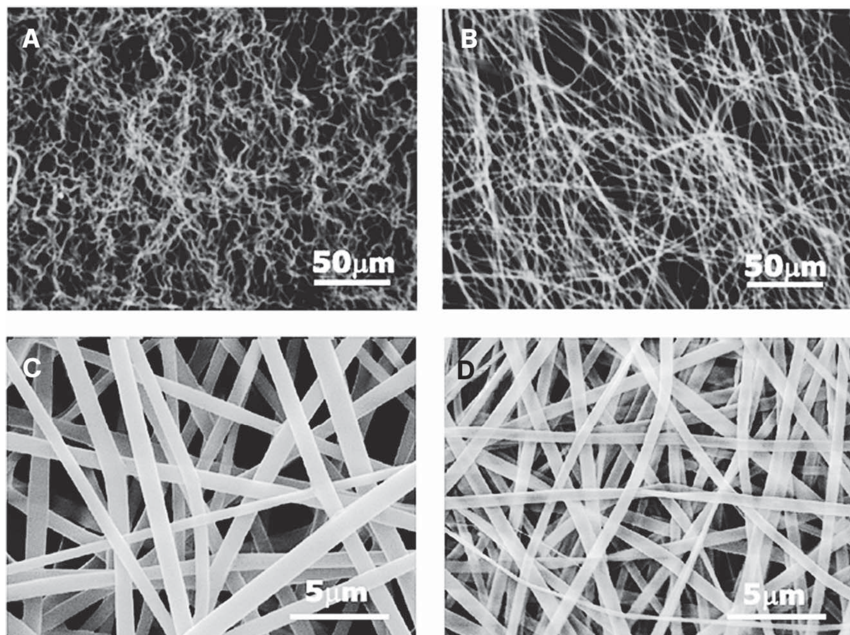

Fig. 1.1 Typical images of scaffolds for tissue engineering. Electrospun elastin (A) and gelatin (B) fibers. Scanning electron microscopy micrographs of electrospun PLGA (C) and PANi-gelatin (D) fibers.