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Aleš Prokop
Yasuhiko Iwasaki
Atsushi Harada *Editors*

Intracellular Delivery II

Fundamentals and Applications

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Editorial and Introduction

This book features a special subsection of Nanomedicine, an application of nanotechnology to achieve breakthroughs in healthcare. The Nanomedicine exploits the improved and often novel physical, chemical, and biological properties of materials only existent at the nanometer scale. As a consequence of small scale, nanosystems in most cases are efficiently uptaken by cells and appear to act at the intracellular level. Nanotechnology has the potential to improve diagnosis, treatment and follow-up of diseases, and includes targeted drug delivery and regenerative medicine; it creates new tools and methods that impact significantly existing conservative practices. This book more specifically targets using nanotechnology in the area of drug delivery and tissue engineering, i.e., the application of various nanoparticulates based on natural or synthetic, organic or inorgarnic materials as drug carriers and tissue regenerative support, first of all to deliver substances and drugs inside cells.

During the last decade, intracellular drug delivery has become an emerging area of research in the medical and pharmaceutical field. Many therapeutic agents can be delivered to a particular compartment of a cell to achieve better activity. In Volume 1 of this series, we investigated various means of delivering cargo, via endocytosis. Various carriers have been investigated for efficient intracellular delivery, either by direct entry to cytoplasm or by escaping the endosomal compartment. These include cell-penetrating peptides, and carrier systems such as liposomes, cationic lipids and polymers, polymeric nanoparticles, etc. Various properties of these carriers, including size, surface charge, composition, and the presence of cell-specific ligands, alter their efficacy and specificity toward particular cells. Also included were various aspects of targeted intracellular delivery of therapeutics including pathways, mechanisms, and approaches.

This Volume 2, a continuation of Volume 1 (not numbered this way), is a collection of **authoritative reviews**.

The Part I of this volume deals with *Novel Nanocarrier Design and Processing*, listing some new designs and chemistry. The very first chapter deals with a survey of production methods of nanofibers, as exemplified by properietary and succesfull Nanospider^{TM} technology developed by Technical University Liberec, Czech Republic, licensed to Elmarco (Liberec, Czech Republic) (www.elmarco.com). This technology has also been licensed in several countries with applications in different fields as well as in biomedicine. It should be stressed that nanofibers are

readily taken up by cells (e.g. Che et al. 2011). Other four chapters describe several different new designs for nanoparticles, with emphasis on responsiveness to different external stimuli.

Part II deals with *Nanocarrier Characterization and Function* The first chapter of this section describes, in some details, how nanoparticles (NP) enter the cells and how they are distributed within the cell interior, while the subsequent chapter describes specific problems related to delivery to mucus. Following are two chapters which cover rather physical methods of nanocarrier characterization, the rest of this section introduces novel delivery vehicles for specific sites or specific cargo.

Part III is entirely a new section; it covers *Simulation for Delivery and Function*. Future applications in nanotechnology are likely to require this level of sophisticated control in order to form precisely ordered structures, with specific chemical and physical properties. Theoretical understanding of the fundamental principles of self-assembly and the design rules for creating new self-assembling materials.

Based on a paper by Vauthier and Bouchemar (2009) two out of about ten different methods of nanoparticle production are (a) formation of polyelectrolyte complexes and (b) production of nanogels. Self-assembly processes typically, both or colloidal building blocks above combine spontaneously to form ordered structures and that without guidance or control from an outside source. Resulting from a disordered system of pre-existing components is an organized structure or pattern as a consequence of specific, local interactions among the components themselves. Self-assembly can be classified as either static or dynamic process. In *static* self-assembly, the ordered state forms as a system approaches equilibrium (thermodynamic stability). In *dynamic* self-assembly, patterns of pre-existing components organized by specific local interactions are not commonly described as "self-assembled" (characterized by the presence of long-range repulsive and short-range attractive forces), whereas they should, in fact, be denoted as "self-organized" (Wikipedia).

New computational simulation tools are required to describe the self-assembly, and to apply them to understand the structures and their thermodynamics and dynamics of both biological and synthetic self-assembling systems (Frenkel and Smit 2002). We envisage that in the future it would be possible to tailor nanoparticles to deliver cargoes at the right subcellular compartment through the use of signaling signatures and pathways. This will improve the magnitude and duration of the drug effects. It is a challenging task due to the complexity of multiple compartments such as endosomes and nuclei, which themselves are dynamic and can undergo fusion and fission and exchange their content (Csukas et al. 2011). The result is to guide further experimental efforts in determining most sensitive parameters. Moreover, there is still much room for building knowledge about the interactions of NPs with proteins and membrane structures on the cell surface. Taking advantage of computer simulations and current developments in interactomics, it would certainly be of great use to know the molecules that interact with the NPs, as well as the nature of this interaction. We emphasize this effort as the literature is relatively scarce in this direction.

The last chapter of this section seeks to emphasize an importance of theoretical background, as provided by Systems Biology, to guide the researcher in the process of discovery. That is, guide the drugs/reagents to an appropriate site. Targeting, localized and intracellular delivery present still a key challenge to effective delivery. To establish an effective fight against diseases, we have to have the ability to selectively attack specific cells, while saving the normal tissue from excessive burdens of drug toxicity. However, because many drugs are designed to simply kill specified cells, in a semi-specific fashion, the distribution of drugs in healthy organs or tissues is especially undesirable due to the potential for severe side effects. Consequently, systemic application of these drugs often causes severe side effects in other tissues (e.g., bone marrow suppression, cardiomyopathy, neurotoxicity), which greatly limits the maximal allowable dose of the drug. In addition, rapid elimination and widespread distribution into nontargeted organs and tissues requires the administration of a drug (in a suitable carrier) in large quantities, which is often not economical and sometimes complicated due to nonspecific toxicity. This vicious cycle of large doses and the concurrent toxicity is a major limitation of many current therapies. Thus, the benefit of nanocarrier design.

Part IV covers *Nanocarriers for Drug Discovery and Treatment*, listing specific applications in biology and medicine. Of a special interest should be a proprietary technology of Contipro s.r.o. (Dolni Dobrouc, Czech Republic; www.contipro.com) employing low-molecular weight hyaluronate to assemble highly biocompatible nanofibers using a technology based on needle-less electrostatic filament principle. The company's main emphasis is in wound healing and other applications.

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The Editors would like to profoundly thank all contributors to this volume for their cooperation and enthusiasm, and also, for their reviewing of colleagues' chapters, which served as a basis of internal review process. Finally, we invite contributions from different researchers to this series.

In future volumes, the emphasis will be more on pharmacokinetic aspects as they control the ultimate application and utility. As pointed by Karel Petrak (personal communication on 10/28/2013), "Although I understand the importance of having 'enabling technology' available, the issue of 'promises, promises and more promises' being made about 'new delivery systems' that are never delivered only to be replaced by new promises. To me the central issue is to recognize that the systems must focus on modifying the drug's pharmacokinetics and pharmacodynamics to be optimal for the given disease target." This volume, unfortunately, does not spell out this emphasis clearly. Thus, this eminent topic is sought for future volumes.

Aleš Prokop
Yasuhiko Iwasaki
Atsushi Harada

Part I
Novel Nanocarrier Design and Processing

Proprietary Nanofiber Technologies and Scale-Up

Stanislav Petrík

Abstract An overview of scalable methods for industrial production of nanofibers is given. The theoretical principles of both nozzle- and nozzle-less electrospinning processes are discussed. Productivity limits of electrospinning and competing/complementary technologies (nano-meltblown, force-spinning, islets-in-the sea), together with their predominant potential application areas, are described. Newest developments in production methods for nanofibers are introduced, e.g. nozzle-less co-axial electrospinning and single-nanofiber preparation.

Keywords Nanofibers · Electrospinning · Co-axial · Nozzle-less · Drug delivery · Bio-medical · Force spinning · Nanofiber production

Abbreviations

Φ	Scalar velocity potential
p	Hydrostatic pressure
ρ	Liquid density
E_0	Electric field strength
γ	Surface tension
ω	Angular frequency
k	Wave number
E_c	Critical electric field intensity
λ	Spatial period ("wavelength")
a	Capillary length

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1 Introduction

Nanofibers attract consistently growing attention for many applications, including bio-medical, since recent decade. Unique morphology of nanofibers, their extremely high surface area, material variability and relatively simple methods for their preparation opened huge field for both technology processes and material applications research. Number of publications related to the use of nanofibers as delivery systems exhibit probably the highest growth during last few years (Yu et al. 2009).

Electrospinning as a method for production of very fine (submicron) fibers has developed into a dominant technology of industrial production scale. Some limitations connected with the use of (often dangerous) solvents and relatively low productivity for some applications motivate developments of alternate methods which are being commercialized during recent years.

Electrospinning methods for creating nanofibers from polymer solutions have been known for decades (Kirichenko et al. 2007; Ramakrishna et al. 2005). The nozzle-less (free liquid surface) technology opened new economically viable possibilities to produce nanofiber layers in a mass industrial scale, and was developed in the past decade (Jirsak et al. 2005; Petrik and Maly 2009). Hundreds of laboratories are currently active in the research of electrospinning process, nanofiber materials, and their applications. Nanofiber nonwoven-structured layers are ideal for creating novel composite materials by combining them with usual nonwovens. The most developed application of this kind of materials is air filtration (Jaroszczyk et al. 2009). Liquid filters and separators are being developed intensively with very encouraging results. Inorganic/ceramic nanofibers attract growing interest as materials for energy generation and storage (solar and fuel cells, batteries), and catalytic materials (Kavan and Grätzel 2002; Rubacek and Duchoslav 2008; Bognitzki et al. 2001).

To fully explore the extraordinary number of application opportunities of nanofibers, the availability of reliable industrial-level production technology is essential. This chapter intends to demonstrate that some of the technologies have matured to this stage.

2 Nanofibers as Delivery Systems

Well known are several bio-medical applications utilizing nanofiber materials, often from biocompatible/degradable polymers like PLA, gelatine, collagen, chitosan. These developing applications include wound care, skin-, vessel-, bone-scaffolds, drug delivery systems and many others (Proceedings 2009).

One of the first reports about electrospinning nanofibers as delivery systems was published by Kenawy et al. (2002) Electrospun fiber mats were explored as drug delivery vehicles using tetracy-cline hydrochloride as a model drug. The mats were made either from poly (lactic acid) (PLA), poly (ethyl-ene-co-vinyl acetate)

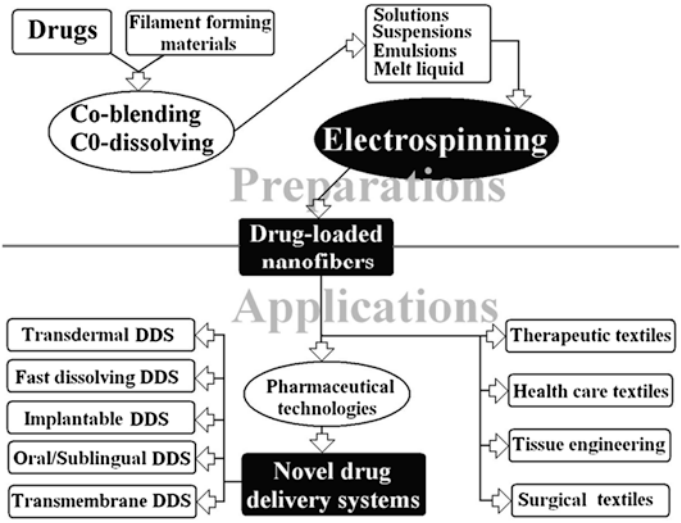

Fig. 1 Applications and preparations of electrospun drug-loaded nanofibers (Yu et al. 2009) (Courtesy of Scientific Research Publishing)

(PEVA), or from a 50:50 blend of the two from chloroform solutions. A detailed overview of delivery bio-medical applications of nanofibers was published by Yu et al. (2009). Their schematic diagram (Fig. 1) illustrates most of the opportunities the nanofiber systems offer for drug delivery, scaffold/tissue engineering, health care textiles, surgical textiles, and other systems.

The active agents (i.e. drugs) can be incorporated into nanofibers in several ways. The most common one used to be to mix functional particles into the polymer solution the nanofiber material is being prepared from. This approach often limits technological processability of the material. As many authors have proven (Buzgo et al. 2013; Mickova et al. 2012; Williams et al. 2012), co-axial (core-shell) nanofibers offer much larger potential as delivery systems, because of their capability to incorporate and protect also the agents which are not spinnable or non-dispersable in homogeneous nanofibers. Besides „trivial"technological approach based on co-axial needle electrospinning (i.e. Azarbayjani et al. (2010)), Lukas'group at the Technical University of Liberec (Vyslouzilova et al. 2010) has patented and published a nozzle-less productive electrospinning device described below.

3 Electrospinning

The electrospinning process is an interesting and well-characterized physical phenomenon and has been an attractive subject for theoretical investigations of several groups (Bognitzki et al. 2001; Doshi and Reneker 1995; Thompson et al. 2007;

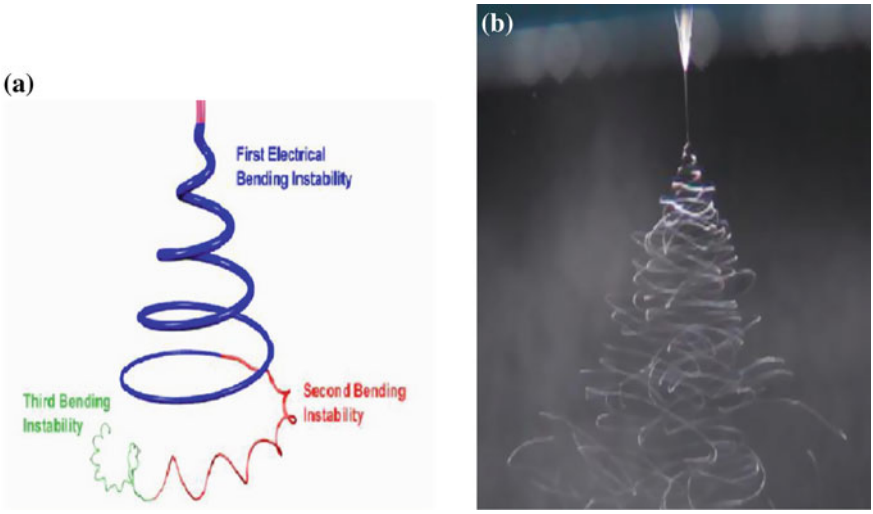

Fig. 2 The path of an electrospinning jet **a** schematic, **b** stroboscopic photograph (*Courtesy of Darrell Reneker, University of Akron*)

Shin et al. 2001; Yu et al. 2006; Hohman et al. 2001). Most work concentrates on the essentials of the process—the nanofiber formation from a liquid polymer jet in a (longitudinal) electric field. It has been theoretically described and experimentally proven that the dominant mechanism is whipping elongation occurring due to bending instability (Thompson et al. 2007; Yu et al. 2006; Hohman et al. 2001). Secondary splitting of the liquid polymer streams can occur also (Kirichenko et al. 2007), but the final thinning process is elongation.

In Fig. 2, the schematic of bending mechanism derived from physical model (a) is compared with a stroboscopic snapshot (b) (Reneker 2009).

A comprehensive analysis (electrohydrodynamic model) of the fiber formation mechanisms published by (Hohman et al. 2001) describes the regions of individual kinds of instability observed during the process. It has predicted and experimentally proven that there is a domain of the process variables where bending instability dominates, as illustrated in Fig. 3.

The efforts to scale up the electrospinning technology to an industrial production level used to be based on multiplication of the jets using multi-nozzle constructions (Kirichenko et al. 2007).

In Fig. 4, the multi-nozzle spinning head developed by NanoStatics Company is shown. The principle is based on an idea to feed multiple nozzles from a single source of the polymer solution.

Figure 5 shows the multi-nozzle spinning part of the machine being commercialized by TOPTEC Company. The device uses upwards direction of electrospinning in order to eliminate polymer droplets eventually falling from conventional down-oriented electrospinning elements.

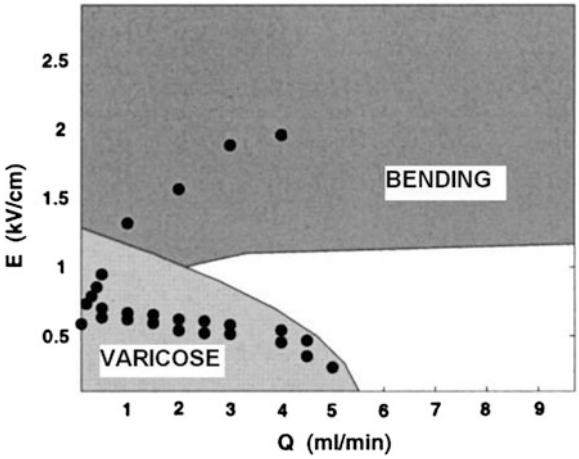

Fig. 3 Operating diagram for a PEO jet. The *upper shaded* region shows the onset of the whipping instability, the *lower* one shows the onset of the varicose instability (Hohman et al. 2001b)

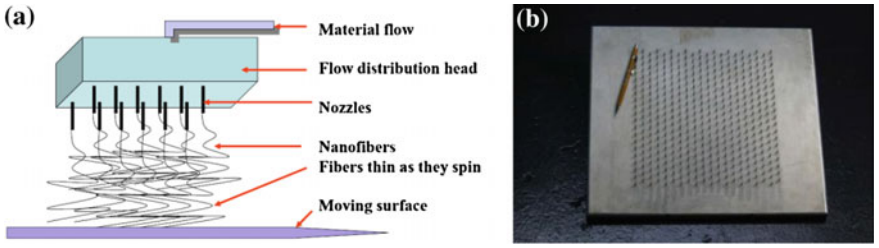

Fig. 4 Schematic (**a**), and photograph (**b**) of a multi-nozzle spinning head by NanoStatics (NanoStatics 2007)

However, the number of jets needed to reach economically acceptable productivity is very high, typically thousands. This brings into play many challenging task, generally related to reliability, quality consistency, and machine maintenance (especially cleaning). The nozzle-less electrospinning solves most of these problems due to its mechanical simplicity, however, the process itself is more complex because of its spontaneous multi-jet nature. The study by (Lukas et al. 2008) focused on the process of multi-jet generation from a free liquid surface in an electric field. They derived an expression for the critical spatial period ("wavelength")—the average distance between individual jets emerging from the liquid surface (Fig. 5). In this system, self-organization of the jets occurs, thus the number and spacing of the jets is optimal even if the technology variables (voltage, viscosity and surface tension of the solution) change. This feature leads to significant improvement of the process stability and consistent quality of the produced nanofiber layer.

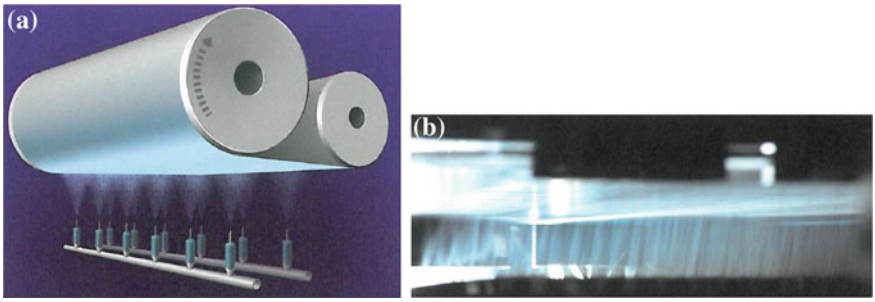

Fig. 5 Schematic (**a**), and photograph (**b**) of a multi-nozzle spinning head by TOPTEC (TOPTEC 2011)

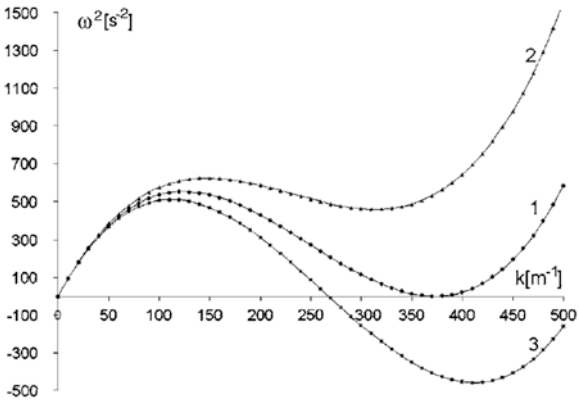

Fig. 6 Relationship between the square of the angular frequency and the wave number for distilled water, electric field is the parameter *1* $E = E_c = 2.461\,945\,094 \times 10^6$ V/m, *2* $E = 2.4 \times 10^6$ V/m, and *3* $E = 2.5 \times 10^6$ V/m (Lukas et al. 2008) (Courtesy of D. Lukas, TU Liberec)

The study showed that the process can be analyzed using Euler's equations for liquid surface waves

$$\nabla\left(\rho\frac{\partial\Phi}{\partial t}+p\right)=0 \tag{1}$$

where Φ is the scalar velocity potential, p is the hydrostatic pressure, and ρ is the liquid density. They derived the dispersion law for the waves in the form

$$\omega^2=\left(\rho g+\gamma k^2-\varepsilon E_0^2 k\right)\frac{k}{\rho} \tag{2}$$

where E_0 is electric field strength, γ—surface tension.

The relationship between angular frequency ω and wave number k is in Fig. 6, electric field is the parameter. When a critical electric field intensity is reached (E_c, curve 1), ω^2 is turned to be negative, ω is then a purely imaginary value, and hence, the amplitude of the liquid surface wave

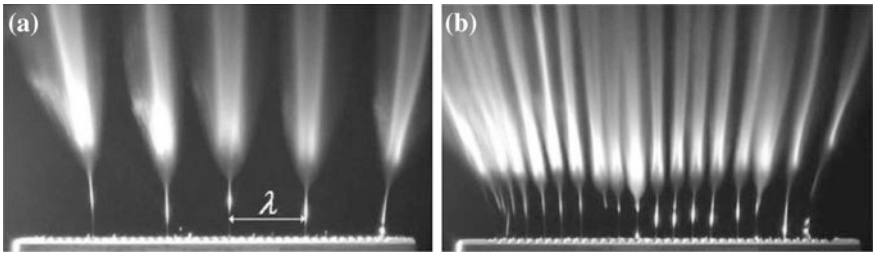

Fig. 7 **a** Free liquid surface electrospinning of Polyvinyalcohol at 32 kV, and **b** 43 kV (*Courtesy of David Lukas, Technical University of Liberec*)

$$\xi = Ae^{qt}\exp(ikx) \tag{3}$$

exponentially grows, which leads to an instability.

Critical field strength can then be expressed

$$E_c = \sqrt[4]{4\gamma\rho g/\varepsilon^2} \tag{4}$$

From this equation, they derived the expression for the critical spatial period ("wavelength")—the average distance between individual jets emerging from the liquid surface (Fig. 7).

$$\lambda_c = 2\pi/k_c = 2\pi a \tag{5}$$

and

$$\lambda = 12\pi\gamma/\left[2\varepsilon E_0^2 + \sqrt{\left(2\varepsilon E_0^2\right)^2 - 12\gamma\rho g}\right] \tag{6}$$

a is the capillary length

$$a = \sqrt{\gamma/\rho g} \tag{7}$$

The simplest realization of the nozzle-less electrospinning head is in Fig. 8a. A rotating drum is dipped into a bath of liquid polymer. The thin layer of polymer is carried on the drum surface and exposed to a high voltage electric field. If the voltage exceeds the critical value, a number of electrospinning jets are generated. One of the main advantages of nozzle-less electrospinning is that the number and location of the jets is set up naturally in their optimal positions. In the case of multi-needle spinning heads, the jet distribution is made artificially. The mismatch between "natural" jet distribution and the real mechanical structure leads to instabilities in the process, and to the production of nanofiber layers which are not homogenous.