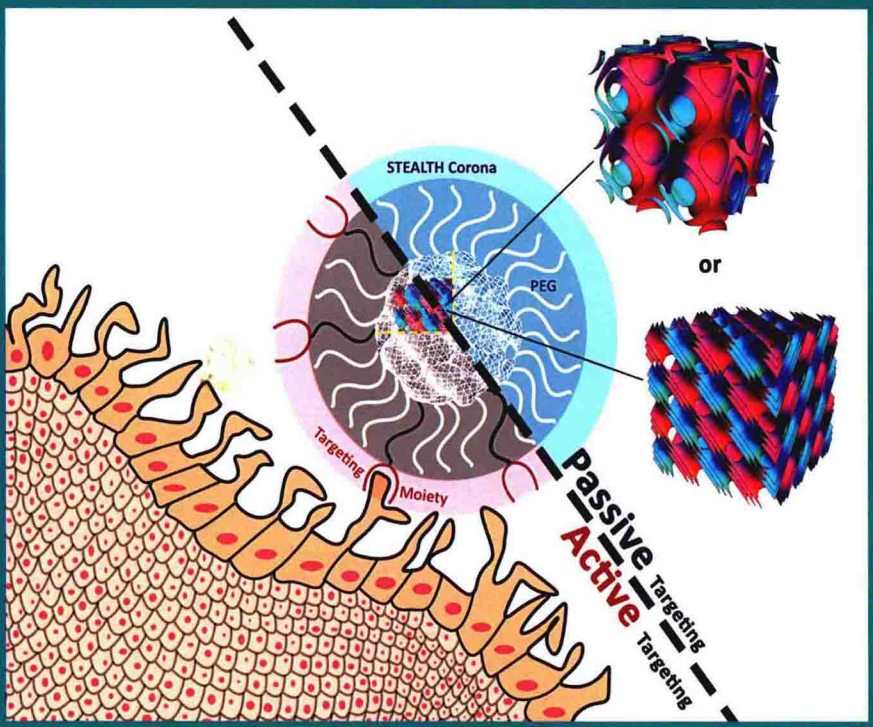
Advances in

Planar Lipid Bilayers and Liposomes

Volume 21



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VOLUME TWENTY ONE

ADVANCES IN PLANAR LIPID BILAYERS AND LIPOSOMES

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Academic Press is an imprint of Elsevier

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225 Wyman Street, Waltham, MA 02451, USA
525 B Street, Suite 1800, San Diego, CA 92101-4495, USA
125 London Wall, London, EC2Y 5AS, UK
The Boulevard, Langford Lane, Kidlington, Oxford, OX5 1GB, UK

First edition 2015

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ISBN: 978-0-12-802116-3
ISSN: 1554-4516

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PREFACE

Once again, *Advances in Planar Lipid Bilayers and Liposomes* (*APLBL*) is embracing with its 21st volume a wide spectrum of applied lipid model membranes. Most of the presented artificial systems are designed for highly specialized purposes and thus have been assembled with great care and developed over many years. Moreover, while the standards of functionality are constantly progressing, and not all model aspects can be tested theoretically, in the end these novel—often composite—model systems can only prove their worth under practical conditions. This volume includes five chapters reporting on exciting new trends in the field of applied model membranes and is highlighting latest developments on polymer/nanodiamond coatings for enhanced cell growths, different tethered phospholipid bilayer membranes for the study of protein/lipid interaction, the understanding of apoptosis with model membranes, investigating antimicrobial peptides with giant vesicles, and new steric stabilizers to be used for design of liquid crystal nanodispersions. The content of this volume is potentially useful for a wide scientific community working on model lipid systems focusing on the biotechnological implications such as for the development of unique drug delivery systems and medical imaging agent delivery, the design of new antibiotics, or the better understanding of protein/lipid interactions. We would like to thank all authors who contributed their chapters to the Volume 21—Natalia Krasteva, Gintaras Valincius, Ana Garcia-Saez, Karin Riske, Ben Boyd, and their coauthors. We would like to thank all members of the Editorial Board. We also thank our Technical and Publishing Team of *APLBL* Volume 21, especially Shellie Bryant, Kate Newell, and Omer Mukthar.

Aleš Iglič, Chandrashekhar V. Kulkarni, and Michael Rappolt

CONTENTS

CHAPTER ONE

Development of Polymer/Nanodiamond Composite Coatings to Control Cell Adhesion, Growth, and Functions

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Contents

Abstract

The identification of biomaterials that support appropriate cellular attachment, proliferation, and functions is critical for tissue engineering and cell therapy. There is a growing interest in functional organic/inorganic composites where a small amount of

Advances in Planar Lipid Bilayers and Liposomes, Volume 21
ISSN 1554-4516
http://dx.doi.org/10.1016/bs.adplan.2015.01.001

nanometer-sized material yields better physicochemical properties for cells to attach, grow, and differentiate. In this work, we prepared polymer/nanodiamond composite layers based on hexamethyldisiloxane and detonation-generated nanodiamond (DND) particles, in which the particles were either embedded into a polymer matrix or deposited on the preliminary formed plasma-polymerized (PP) layer. The surface properties of composites, such as roughness and wettability, as well as adhesion, growth, and functions of osteosarcoma MG-63 cells and primary rat mesenchymal stem cells were studied. We aimed to investigate the influence of the incorporation methods of DND into the polymer on the material surface properties and the cell response in order to control them by manipulating diamond-containing composite surfaces. We found differences between both composites in respect to their physicochemical properties and to the cell behavior suggesting that the method of particle incorporation into polymers should be taken in account during the development of new biomaterials for a specific application.

1. INTRODUCTION

Bone tissue-engineered constructs have a great potential for the treatment of large bone defects caused by tumor, injury, or posttraumatic osteomyelitis [1,2]. In such constructs, the key elements are biomaterials that have to provide an appropriate microenvironment for tissue cells to attach, grow, and differentiate [3,4]. It is also crucial the biomaterial to have mechanical properties similar to the native bone [5]. Despite the remarkable progress in recent years, the engineering of materials matching both the mechanical and biological properties comparable with those of natural, healthy bone still remains a challenge.

Composite materials are of interest because they combine the advantages of different materials to achieve specific structural properties, while a single type of material usually cannot provide all necessary properties [6]. From the biological point of view, it makes sense to combine polymer and inorganic compounds to fabricate biomaterials for bone tissue engineering, because human bone tissue is a biologically and chemically bonded composite of inorganic hydroxyapatite embedded in an organic matrix of collagen and noncollagenous proteins [7,8]. The first step toward the development of composites for bone substitution is the identification of the relevant class of biomaterials. A variety of natural and synthetic polymers are now available for bone tissue engineering applications but all of them have some deficiencies [9,10]. Synthetic polymers have gained a significant advantage over naturally occurring polymers because they can be produced under controlled

conditions and therefore their properties are in general predictable and reproducible [11,12]. One class of synthetic polymers widely used in biomedical applications is organosilicones due to their excellent inertness, flexibility, smoothness, and thermal and oxidative stability [13]. Organosilicones have been used for the production of oxygen masks, teats for baby bottles, tubes for extracorporeal circulation in heart surgery and dialysis, drains and catheters, prosthesis, contact lens, insulation coating for leads and circuits, and protective sheaths for pacemakers [14]. The low mechanical stability of organosilicones limits their application as heavy load-bearing bone substitutes. However, they can be used for deposition of thin coatings onto bone implants to improve cell-contacting properties of implants' surface. For the preparation of such coatings of great interest are organosilicones, obtained by plasma polymerization. Plasma polymerization allows deposition of high-dense, pinhole-free, and well-adherent films on a variety of substrates like conventional polymers, glass, and metals. Other advantages of the plasma polymerization process are the easy varying of process parameters and the use of modificators and fillers to produce new materials and composites with stable properties [15].

On the other side, a member of the nanocarbon family, detonation nanodiamond (DND), has emerged recently as a novel promising material for biological applications [16–22]. The nanoscale diamond material is chemically robust, nontoxic at both cellular and organism levels, and easily functionalized with different macromolecules [23–25]. Therefore, nanodiamonds can be used as reinforcements or additives in various materials to increase mechanical stability and to improve tissue interactions [26–28]. One advantage of nanoparticles as polymer additives compared to traditional additives is that the loading requirements are quite low, meaning that a small amount of nanoparticles is necessary to alter properties of materials. The inclusion of only a few percent of nanosized diamond particles into a polymer matrix may increase the stiffness and strength of the polymers and may also create nanotopographic features that mimic the nanostructure of bones. The properties of polymer–nanodiamond composites can be easily tailored by changing the type, concentration, and size of nanoparticles. However, the incorporation of particles into the polymer matrix strongly influences the bonding between particles and polymers and thus the properties of the obtained composite material [29]. Currently, there is not enough information concerning how the incorporation of nanodiamond particles into a siloxane matrix affects the surface properties of siloxane–nanodiamond composites and how this can be used to control

the osteoblast and mesenchymal stem cell behavior for the purpose of bone tissue engineering.

In this study, we addressed the need for the development of methods targeted at composite layers based on plasma-polymerized hexamethyldisiloxane (PPHMDS) and DND particles as polymer modificators. The aim was to correlate the incorporation approach with physicochemical characteristics of composite layers and to characterize the cell behavior of osteoblastic cell line (MG-63) and rat mesenchymal stem cells (rMSCs). The different cell models were used in order to elucidate if the biological response to composites is more material-specific than cell-specific.

The surface roughness and the wettability of plasma-polymerized (PP) layers were evaluated by using the atomic force microscopy (AFM) and contact angle measurements. The cell adhesion was characterized by studying the overall cell morphology, cell attachment and spreading, and actin cytoskeleton organization. In addition, the cell proliferation and the alkaline phosphatase (ALP) activities of both cell types were measured. Different cell models were used to elucidate if the biological response to composites is more material-specific than cell-specific. The effect of fibronectin (FN) preadsorption of materials on cell behavior was studied.

2. MATERIALS AND METHODS

2.1 Synthesis of polymer/nanodiamond composite layers

Two different composite DND/PPHMDS materials and one pure polymer, PPHMDS, were synthesized, following the method of plasma polymerization. For the preparation of polymer PPHMDS layers and the composite layers (DND/PPHMDS), hexamethyldisiloxane (HMDS; Merck, Germany) with purity >99.99% was used. The detonation-synthesized nanodiamond powder was obtained from detonation soot, produced by SRTI-BAS (Sofia, Bulgaria), with the subsequent purification from non-diamond carbon and metal impurities through oxidation with potassium dichromate in sulfuric acid and refinement with HNO_3 and HCl [30].

For the preparation of DND/PPHMDS composites, two approaches for the incorporation of DND particles into the siloxane matrix were applied: (1) the plasma deposition of preliminary prepared mixture of monomer (HMDS) and DND nanoparticles by the method described in Ref. [28]. This group of samples is denoted as 1-DND/PPHMDS; (2) the plasma deposition of DND particles onto the preliminary plasma-deposited polymer layer (PPHMDS). This second group of samples, denoted as

2-DND/PPHMDS, was prepared by the deposition of PPHMDS on cover glass (CG) as a first layer with a current density of 0.16 mA/cm^2 and monomer (HMDS) flow rate 10 L/h for 10 min, and a second layer obtained from DND particles suspension in ethanol on the PPHMDS layer at a current density 0.16 mA/cm^2 for 10 min. After the deposition, the samples were washed with deionized water and air-dried. The composite and the PPHMDS layers were deposited on commercially available CG (Menzel Glaeser) with a diameter of 15 mm.

2.2 Surface characterization of the composite layers

In order to obtain information about the surface topography and hydrophilicity of the prepared polymeric layers, all samples were analyzed with the atomic force microscope (AFM, Solver Pro; NT-MDT, Russia) and a sessile drop method (Easy Drop FM40; Kruss, Germany) under ambient condition. Contact angles were then calculated with the computer software (DSA1; Kruss, Germany). At least five different spots on each sample were selected for the measurements.

2.3 Cell culture experiments

2.3.1 Cell cultures

rMSCs were isolated from the bone marrow of femurs and tibias of 4-week-old male rats according to the centrifugation method of Dobson *et al.* [31]. Briefly, femur and tibia were cleaned out of the surrounding tissue and the phosphate-buffered saline (PBS) was flushed out of the bone marrow. The obtained cells were centrifuged and the pellet was resuspended in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Sigma, Germany) and 1% streptomycin/penicillin (Sigma, Germany). Cells were seeded into a cell culture flask (Costar; Capitol Scientific, Inc., TX, USA) and were incubated at 37 °C in 5% CO_2 for 72 h. At the end of incubation, the medium was discarded to remove nonadherent cells. Experiments were performed with cells between passages 3 and 7.

Human osteosarcoma cells (cell line MG-63) were purchased and characterized by American Type Culture Collection. Experiments were performed with cells between passages 23 and 26.

Both cell types were cultured in DMEM (Sigma-Aldrich, Germany), supplemented with 10% FBS, 1% streptomycin/penicillin mixture, 2 mM L-glutamine (Roche Diagnostics, Germany), and 1 mM sodium pyruvate

(Gibco BRL, Scotland) in the incubator at 37 °C, with 5% CO_2 and high air humidity. The cells were harvested every second day.

When the cells reached 70–80% confluency, they were detached from the polystyrene cell culture flask with 0.05 mM trypsin/0.25% EDTA solution and used for experiments. The cells were resuspended in DMEM in the absence of the serum and seeded onto polymer films with the concentration 20,000 cells/ml or 10,500 cells/cm^2. At different time points, the samples were removed and processed in accordance with the type of cell experiments.

2.3.2 Cell adhesion assay

Cell adhesion was analyzed qualitatively through observation of the attached cells' morphology by using optical and scanning electron microscopy (SEM) and quantitatively with estimation of the number and average spreading area of the attached cells. Adhesion experiments were conducted in a serum-free medium because the presence of various adhesive factors in the serum can mask the effect of surface properties. Prior to the experiment, some of the materials were precoated with FN with a concentration of 20 μg/ml for 30 min at room temperature (RT). The cells were seeded onto the different substrates: CG, PPHMDS and both DND/PPHMDS composite layers, and were incubated for 2 h in a serum-free medium. At the end of incubation, the nonattached cells were removed by a gentle washing with PBS and the images of the adherent cells were captured using a Zeiss Axiovert 25 microscope equipped with a digital camera (InRay Solutions, Bulgaria). The number of attached cells and the spreading area were measured using the image analysis software (ImageJ).

2.3.3 Scanning electron microscopy

After a predefined time of incubation (2 h), the cells were washed three times with PBS and fixed for 2 h at RT using a modified Karnovsky fixative, composed of 2.5% glutaraldehyde (SPI Supplies, West Chester, PA, USA) and 0.4% paraformaldehyde (Merck KGaA, Darmstadt, Germany) in 1 M sodium phosphate buffer ($NaH_2PO_4 \cdot 2H_2O$ and $Na_2HPO_4 \cdot 2H_2O$; all from Merck KGaA, Darmstadt, Germany). After washing in 1 M sodium phosphate buffer (3 × 10 min), a postfixation of samples was undertaken with 1% OsO_4 (2 × 20 min; SPI Supplies, West Chester, PA, USA) and 2,3,5-triphenyltetrazolium chloride (2 × 30 min; SPI Supplies, West Chester, PA, USA). Samples were dehydrated with a series of ethanol and acetone (Merck KGaA, Darmstadt, Germany), with duration of each step 10 min and were dried with hexamethyldisilazane (HMDS; SPI Supplies,

West Chester, PA, USA), which was left to evaporate for 24 h before placing the samples on the aluminum holders. Finally, the samples were sputtered with Au and inspected with SEM.

2.3.4 Immunofluorescent labeling of the actin cytoskeleton

The procedure for labeling the actin cytoskeleton was performed as previously described [32]. Briefly, the cells cultured for 1, 2, and 4 h on plasma films were rinsed with PBS and fixed with 5% paraformaldehyde for 10 min at RT. After a triple wash with PBS, the cells were permeabilized with 0.5% Triton X-100 for 5 min, rinsed with PBS, and blocked with a 1% BSA solution for 30 min at RT. To visualize F-actin, the cells were stained with Alexa Fluor 566 phalloidin (Invitrogen, Sweden) for 30 min at RT, followed by an extensive rinse with PBS. Staining was conducted in the dark to prevent a photobleaching of the fluorochrome. Samples were finally washed in distilled water, embedded in Fluoroshield mounting medium (Sigma-Aldrich, Steinheim, Germany), air-dried, and analyzed under a Zeiss Axiovert 25 invert microscope (Germany). Fluorescence micrographs were photographed at a magnification of ×63, using a digital camera and the Motic Images 2.0 software.

2.3.5 Cell proliferation assay

MG-63 cells and rMSCs were seeded on PP layers, as described above, and incubated for 7 days in FBS-supplemented medium. Cell proliferation was determined on the first and on the seventh day of incubation with a modified lactate dehydrogenase (LDH) assay. LDH assay is a colorimetric method originally developed for the quantification of cell death (Roche Diagnostics, Mannheim, Germany) based on the measurement of LDH activity released from the cytosol of damaged cells. Here, we applied this method to measure the enzyme activity after total cell lysis, thus quantifying the total amount of cells and hence cell proliferation.

The assay was performed according to the manufacturer's instructions. Briefly, at indicated time points the samples were transferred to a new 24-well plate and were rinsed once with PBS containing 150 mM NaCl, 5.8 mM Na_2HPO_4, 5.8 mM NaH_2PO_4, pH 7.4. Then, the attached cells were lysed in 0.5 ml 0.5% Triton X-100 in PBS under shaking for 30 min at RT and 100 µl of each cell lysate was transferred into a 96-well plate. To start the reaction, 100 µl of LDH reaction mix was added to each well, mixed, and incubated for 30 min at RT in the dark. The absorbance was measured with a plate reader (TECAN Infinite, F200 Pro) at 492 and