



Investigation of cell–substrate interactions by focused ion beam preparation and scanning electron microscopy

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ABSTRACT

Cell–substrate interactions, which are an important issue in tissue engineering, have been studied using focused ion beam (FIB) milling and scanning electron microscopy (SEM). Sample cross-sections were generated at predefined positions (target preparation) to investigate the interdependency of growing cells and the substrate material. The experiments focus on two cell culturing systems, hepatocytes (HepG2) on nanoporous aluminum oxide (alumina) membranes and mouse fibroblasts (L929) and primary nerve cells on silicon chips comprised of microneedles. Cross-sections of these soft/hard hybrid systems cannot be prepared by conventional techniques like microtomy. Morphological investigations of hepatocytes growing on nanoporous alumina membranes demonstrate that there is in-growth of microvilli from the cell surface into porous membranes having pore diameters larger than 200 nm. Furthermore, for various cell cultures on microneedle arrays contact between the cells and the microneedles can be observed at high resolution. Based on FIB milled cross-sections and SEM micrographs cells which are only in contact with microneedles and cells which are penetrated by microneedles can be clearly distinguished. Target preparation of biological samples by the FIB technique especially offers the possibility of preparing not only soft materials but also hybrid samples (soft/hard materials). Followed by high resolution imaging by SEM, new insights into cell surface interactions can be obtained.

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1. Introduction

In biomedical research the development of new substrates for cell cultivation and tissue engineering is of great importance. Therefore, the interaction of cells or living tissues with the surfaces of artificial materials is relevant, both in research and practice [1]. These surface interactions can be used to guide the organization, growth and differentiation of cells [2]. Interactions between the material and the biosystem can be influenced by modification of the surface properties, in order to improve the biocompatibility of the substrate. Cell adherence and the formation of focal adhesion points especially have been the focus of such studies [3,4].

Usually these morphological details are observed by light microscopy or laser scanning microscopy. However, these techniques do not allow the direct visualization and observation of the interface between cells and the substrate. Other conventional high resolution microscopical methods with are scanning electron microscopy (SEM) and transmission electron microscopy (TEM), both of which require complex sample preparation and, especially for TEM, effective preparation of cross-sections. In the majority of cases cross-sections are generated with a microtome. Microtomy

or ultramicrotomy are widely used, but are unfavorable if the sample consists of a combination of soft biological and hard brittle materials, like cell cultures on ceramics or silicon. Usually the samples will be damaged during sectioning with a microtome and investigations of the interface between cells and the substrate material are no longer possible. Therefore, focused ion beam (FIB) technology, often referred to as FIB milling, offers a new means of cross-section preparation at preselected sample positions [5–7].

In a FIB workstation a focused gallium ion beam can be used for ablation and deposition of material on the surface of a specimen [8,9]. The resolution of the FIB is <10 nm. Cross-sections of a sample can be prepared with such beams. The secondary ions and electrons generated by the ion beam are also used for surface imaging, but nowadays the FIB source is incorporated into a high resolution scanning electron microscope (dual beam technology). The potential of this technique ranges from top to down structuring (etching or deposition of nanostructures) to three-dimensional (3D) tomographic characterization of complex microstructures and composite materials [10–12]. Further, FIB technology allows effective preparation of TEM lamellae on predefined areas (target preparation), a process that can be simultaneously monitored by SEM.

Although FIB technology is widely used in the semiconductor industry, its usability for the preparation of solid polymer

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interfaces or cross-sectioning of adherent cells on scaffolds is still underestimated [12,13]. As a result of the limited availability of FIB technology so far, only a few studies of FIB processing of such materials have been carried out. In polymer material science FIB preparations are used for surface nanostructuring [12,14–16], as well as to investigate aging effects on polymer components [17]. The use of FIB for the preparation of polymeric samples is delicate, because it can induce amorphization of the sample surface, scission and/or cross-linking of polymer chains, shrinkage of the chains, and modification of the surface chemistry [12,13,18]. To suppress such damaging effects of the ion beam on polymers and biological specimens a well-defined set of processing parameters has to be considered [19,20].

The number of publications in the field of FIB milling of biological samples like cell cultures and tissue material has steadily increased over recent years. Based on the milling strategy, papers can be classified into two categories. On the one hand, FIB milling was used to prepare thin sections (lamella) of biological materials for high resolution TEM imaging [21–23]. On the other, cross-sections of biological samples were prepared by FIB milling and directly investigated by SEM, using, for example, dual beam FIB/SEM instruments [5,7,21,25–29]. The latter technique was used in this study. Before ion beam milling prior sample preparation by plastic embedding, drying or freezing is required in both cases.

In this study we present investigations using different FIB slicing techniques followed by high resolution imaging by SEM to observe cell–substrate interfaces. Two types of cell–material systems were selected. First, the morphology and adhesion of cells on nanoporous alumina membranes made by anodic oxidation will be described. These porous membranes can be applied for indirect coculture of different cell types on both membrane sides to investigate and stimulate cell–cell interactions. Secondly, cell cultures on microneedle chips were investigated. These microneedles are used as miniaturized patch-clamp electrodes [30,31]. Thus a detailed investigation of the coupling between or penetration by sensor needles and cultivated cells is required.

Cells cultured in monolayers on nanoporous alumina or silicon microneedle arrays were prepared by conventional sample preparation for electron microscopy. The structure of the substrate surface as well as the cell morphology were imaged by SEM. Cell adhesion and cell–substrate interactions were characterized by FIB sectioning the samples at predefined positions and subsequent SEM imaging. FIB preparation was carried out using two different techniques, slice by slice preparation and pie slice preparation, to cross-section the target areas, as shown in Fig. 1.

The slice by slice preparation technique is carried out by stepwise material ablation in only one direction to create parallel cross-sections of the sample. During this stepwise process cell–substrate interactions as well as cellular focal adhesion sites can be continuously studied (Fig. 1a). With the resulting stack of images it is possible to generate a 3D reconstruction of the observed object at high resolution. In order to locate hidden objects or subsurface structures, like a microneedle below a cell, pie slice preparation can be applied (Fig. 1b). During this process the cutting direction of the ion beam is changed. With a freely selectable cutting direction specific structures inside the sample or hidden objects can be found and effectively prepared, which is obviously a significant advantage compared with conventional microtomy. In this way we introduce a technique able to obtain new insights into the growth behavior of adherent cells on different substrates.

2. Materials and methods

2.1. Preparation of and cell cultivation on nanoporous alumina membranes

Self-supporting nanoporous alumina membranes were prepared using the well-known anodic oxidation of aluminum [32–36]. The membranes exhibited parallel, open pores which were aligned perpendicular to the membrane surface and showed a narrow pore size distribution. Different electrolytes and anodization voltages were used to obtain self-supporting membranes with various pore diameters and membrane thicknesses [37,38]. In brief, electropolished aluminum plates (150 × 100 mm, 99.99% purity, Hydro Aluminum, Germany) were placed into electrolyte baths and connected as the anode. Anodization was carried out under constant potential conditions at 40 V (4 vol.% oxalic acid, membrane M1) and 150 V (1 vol.% phosphoric acid, membrane M2) for 24 h. Self-supporting nanoporous membranes were obtained by a stepwise voltage reduction, followed by mechanical detachment from the underlying aluminum substrate. For the cell cultivation experiments smaller membrane pieces (diameter 12.5 mm) were produced by laser cutting with an Nd-YAG laser at a wavelength of 1064 nm. The membranes were subsequently immersed in 5 vol.% H₃PO₄ solution for a given time, dependent on the electrolyte used during the anodization process. After cleaning in distilled water the membranes were sterilized in 70 vol.% ethanol for at least 24 h and dried under UV in a laminar flow hood.

The cell culture experiments on nanoporous alumina membranes were performed with the human hepatoma cell line HepG2 (DSMZ GmbH, Germany). The HepG2 cells were cultured in RPMI

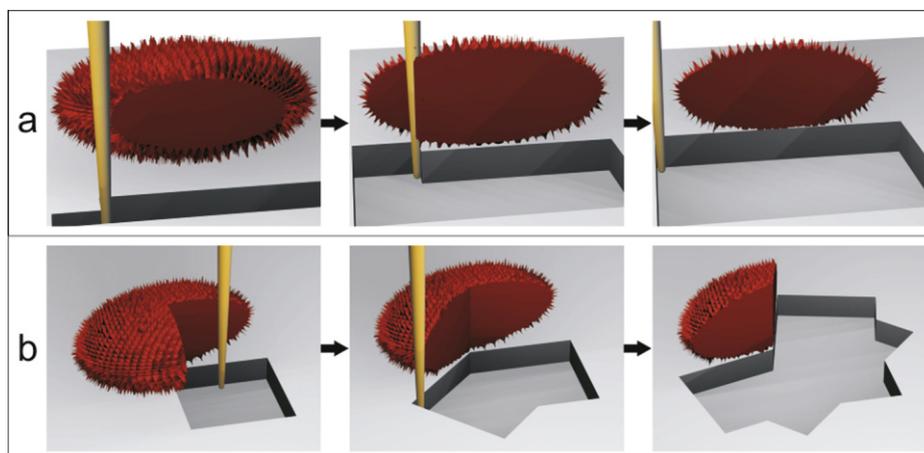


Fig. 1. Schematic diagram of the (a) slice by slice and (b) pie slice FIB preparation techniques.

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