Chapter 1

Cell Colonization Control by Physical and Chemical Modification of Materials

L.Bačáková¹ and V. Švorčík²
¹Department of Growth and Differentiation of Cell Populations, Institute of Physiology, Academy of Sciences of the Czech Republic, 142 20 Prague, Czech Republic,
²Department of Solid State Engineering, Institute of Chemical Technology, 166 28 Prague, Czech Republic

Abstract

Cell-material interface plays a key role in the interaction of cells with artificial materials designed for construction of body implants or tissue engineering. Physical and chemical properties of the material surface, such as its polarity, wettability, electrical charge and conductivity, roughness and morphology, rigidity or elasticity as well as the presence of various chemical functional groups, strongly influence the cell adhesion. The latter is mediated by adsorption of cell adhesion-mediating molecules (e.g. vitronectin, fibronectin, collagen, laminin, fibrinogen) from the serum of the culture medium or body fluids in appropriate spatial conformation or flexibility enabling the accessibility of specific sites on these molecules (e.g., certain amino acid sequences or saccharide-based ligands) by adhesion receptors on the cell membrane (e.g., integrins). After binding these ligands, the adhesion receptors are recruited into focal adhesion plaques, where they associate with cascades of various structural and signalling molecules, such as cytoskeletal proteins and kinases. By these cascades, the signal provided by the cell-material interaction is delivered to the cell nucleus. It influences the gene expression, and thus the further behavior of cells, manifested by their viability, synthesis of various molecules, proliferation, differentiation, functioning, immune activation, stress adaptation or cell death.

This interdisciplinary review involves:
(i) physical modifications of the material surface by plasma discharge, irradiation with ions or ultraviolet light and effects of these modifications on the surface wettability, electrical conductivity and formation of chemical functional groups influencing cell adhesion,
(ii) deposition of organic or inorganic layers on the material surfaces, especially those nanostructured (e.g., nanofibers of fibrin and extracellular matrix molecules, carbon nanoparticles, such as nanodiamonds, fullerenes and nanotubes, nanocomposite metal/C:H layers),

(iii) functionalization of the material surface with amino acids and oligopeptidic ligands for cell adhesion receptors (e.g., GRGDSG),

(iv) effects of all these modifications on the extent and strength of adhesion of vascular and bone-derived cells, cell proliferation activity, switch between cell proliferation and differentiation, expression of cell-type specific markers of differentiation and other cell functions.

1. Introduction

Artificial materials are of growing importance in the fields of medicine and biology. *Tissue Engineering*, a new and modern interdisciplinary scientific field, has been developed to design biocompatible materials in order to substitute irreversibly damaged tissues and organs.

The tissue damage or loss due to disease, inborn defect or trauma is not only a major socio-economic burden on world healthcare systems but leads to a reduced quality of life for the patient. Currently, autogenous tissue, i.e. tissue derived from the same patient, is the most widely used and effective graft material. However, this approach brings a number of related problems, such as availability of the graft material in limited quantity or damage of additional patient’s tissues, prolonged surgical time, blood loss, infection risk and prolonged rehabilitation. Allogenic or xenogenous grafts, i.e. transplants derived from human or animal donors, are associated with a severe risk of immune rejection and disease transmission. Consequently, alternative strategies with potential to overcome these limitations should be generated. One of them is represented by creation of appropriate biocompatible artificial substitutes, which offers enormous promise for the repair of lost tissue function.

In addition, artificial materials have been employed in diverse diagnostic and therapeutic applications and biotechnologies, e.g., tracers for advanced imaging technologies, carriers for controlled drug and gene delivery, biosensors and growth supports for cells in a culture.

Artificial materials designed for the biomedical use should be *biocompatible*, i.e. free of adverse effects on cells and tissues, such as cytotoxicity, immunogenicity, mutagenicity and carcinogenicity. Biocompatible materials can be constructed as *bioinert*, i.e. not allowing adsorption of proteins and adhesion of cells, or as *bioactive*, i.e., promoting colonization with cells - if possible, in a controllable manner. Thus, bioactive materials for advanced tissue engineering are not only simply tolerated by cells but they actively regulate the cell adhesion and growth activity, switch between proliferation and differentiation program, secretion of various molecules by cells, the viability or programmed death of cells and other cell functions. In other words, bioactive materials act similarly as natural extracellular matrix in controlling the cell behavior.

The bioactivity of materials can be enhanced by a wide range of physical and chemical methods. This interdisciplinary review is focused on the modification of materials,
particularly synthetic polymers, by irradiation with plasma, various ions and ultraviolet light, followed by functionalization of the modified material with bioactive molecules, namely amino acids and adhesion oligopeptides. The ligands for cell adhesion receptors were also tethered on degradable materials through flexible polymeric chains, and thus templates mimicking natural extracellular matrix were constructed. In addition, coating material by various soft and hard biocompatible layers, including those nanostructured, is also discussed. The main part of this review is based on our results obtained in the field of biomaterials during more than 10 years.

Figure 1. Principles of plasma interaction with material. The Ar⁺ ions hit polymer surface and create a collision cascade in the surface layer by which a number of atoms are set in movement. As a result ionization of atoms and molecular bond cleavage take place and some of liberated atoms are ejected (sputtering process) [6].

2. Interaction of Plasma Discharge and Energetic Ions with Materials Surface

2.1. Principles of Plasma Treatment [1,2]

Plasma treatment, which is an electric gas discharge, is usually performed in a vacuum chamber in a pressure ranging from 1-100 Pa. Electric field, applied to the gas, accelerates free electrons to energies which are sufficient for ionization, fragmentation and excitation of the gas molecules (see Figure 1). In this way highly reactive gaseous products are produced
which are able to react chemically with exposed surfaces. The processes of the plasma-polymer interaction and the resulting surface properties of the modified polymer are dependent on several factors such as process gas and its properties, chemical structure of polymer and last but not least the properties of the plasma discharge (density and energy of electrons and ions). The nature of the interaction of the plasma constituents with polymers is also affected by plasma reactor configuration. Depending on adjustment of all these factors different outcome of plasma-polymer interaction may result. The most important from the point of view of practical applications are:

(i) **Reactive removal of surface layer** leading to etching or cleaning of the material surface.

(ii) **Activation or more generally modification** of the polymer surface. The plasma treatment changes polymer structure and elemental composition within few molecule layers on the polymer surface and creates new functional groups on the polymer surface. The functional groups may increase adhesion, wettability, bioactivity of polymer surface or provide more inert surface properties.

(iii) **Deposition or plasma polymerisation** is the process when a coating with thickness in sub-micrometer range is deposited onto exposed surface. Generally the coatings exhibit highly cross-linked structure and they provide useful properties for potential applications.

The surface analytical techniques mentioned above provide wealth information on the composition and structure of polymer surface and changes resulting from modification by plasma discharge. It should be however stressed that, despite of broad spectrum of analytical techniques available, the information is not sufficient to understand all underlying processes in their complexity. Especially, it is the case of plasma treatment when the interactions of many plasma constituents with polymer surface may play a role. Existing theoretical models are restricted to some specific cases and they usually describe only some part of the process.

### 2.2. Principles of Ion Implantation [3,4]

Ion implantation is performed in high vacuum (see Figure 2) and, contrary to plasma treatment, there is no complex interaction of reactive gaseous species from ambient atmosphere with material surface. Instead, the polymer modification is caused solely by interaction of energetic ions with polymer matrix. The basic instrumentation consists of an ion implanter, which is commonly a small dedicated accelerator with associated ion optics, beam scanning system and target chamber with a suitable sample holder. The ions are usually accelerated to energies ranging from few keV up to several MeV. The ions are mass separated and send toward the sample. A beam scanning system ensures homogenous irradiation of the sample surface. Indispensable part is a beam monitoring system measuring the total fluence of the ions. The ions entering the polymer substrate interact with atoms which are ionized and exited.
Figure 2. Principles of ion implantation. Basic processes of ion interaction with matter. The thickness of modified surface layer is determined by ion projected range $R_p$, measured in the direction of the sample surface normal.

The ionization and excitation may lead to chemical bond cleavage and production of highly reactive species, free radicals, ions and molecular fragments, which subsequently interact with each other and at last stable degradation products are created. This complex sequence of processes can deliberately be divided into two basic phases, the initial physical phase, in which the ion energy is dissipated to electrons and atoms, and the chemical one comprising interaction of the reactive species and production of the final stable products. Two kinds of degradation products are in principle created, low mass species created by macromolecular chain scission and two- to three dimensional structures produced by macromolecule cross-linking (mediated by free radical reactions).

Specific phenomenon is desorption of light weight, volatile products in the course of the ion implantation. Since the desorbed species are hydrogen-enriched, the polymer surface is progressively carbonized. The thickness of the polymer surface layer modified by implanted ions is determined by ion projected range which is an increasing function of the ion energy and varies, depending on the ion mass and energy and the substrate composition, from several nanometers to tens of micrometers. Typical ion fluences vary from $10^9$ to $10^{18}$ cm$^{-2}$. It should be mentioned that the polymer modification by ion bombardment is not laterally homogenous at least during initial phases of implantation. In fact the modification takes place only in discrete regions along the trajectories of individual ions (ion latent tracks). Once the ion fluence is increased, the sample surface is progressively covered with the ion tracks and at still higher fluences an overlapping of the ion tracks occurs, i.e. the same place is hit by more than one
ion. The properties of the modified polymer could also be influenced by implanted atoms which remain embedded in the polymer matrix.

2.3. Principles of Photochemical Modification by Ultraviolet (UV) Light

UV irradiation is applied typically on surfaces on synthetic polymers, such as polytetrafluoroethylene (PTFE) or polyethylene terephthalate (PET), i.e. materials often applied in biology and medicine, e.g. for construction of clinically used vascular prostheses. Principles of photochemical modification of PTFE by ultraviolet light in ammonia or acetylene atmosphere see in Figure 3 [5]. The pristine polymers show either very low cell adhesion (PTFE) or medium cell adhesion (PET), but exposure to UV-light at wavelengths $\lambda < 200$ nm in a reactive ammonia (NH$_3$), acetylene (C$_2$H$_2$) and oxygen (O$_2$) atmosphere can result in a drastic change of the surface properties, especially in a higher wettability with the first two gases. Treatment in oxygen can also result in etching. The high wettability of treated surfaces is ascribed to polar groups incorporated into the PTFE surface by substitution of F atoms. The subtraction of F is mediated via atomic H which is generated in the dissociation reaction NH$_3$ + h$\nu$ → NH$_2$ + H [6].

Modified PTFE surfaces show a high degree of biocompatibility with good cell adhesion and proliferation [7-11]. However, the UV-treatment results also in a loss of mechanical stability due to the scission of polymer chains, especially for light-sources with wavelengths below 193 nm [6]. Similarly to the ion implantation or plasma modification, also the UV light-irradiation is performed on both sides of a polymer foils in order to avoid the material torsion.

(a) surface reactions (b) reactions in atmosphere (c) reactions in polymer

![Figure 3. Principles of photochemical modification of polymer (e.g. PTFE) by ultraviolet (UV) light in ammonia or acetylene atmosphere (A-B). Basic processes of photochemical modification of polymer by UV light (h$\nu$) in atmosphere are: (a) surface reactions, (b) reactions in atmosphere and (c) reactions in polymer. [5].]
3. Physical and Chemical Characterization of the Modified Material Surface

Several techniques can be used for characterization of the physical and chemical properties of pristine polymers and polymers modified by various chemical and physical treatments as well. The techniques can deliberately be divided in two groups, those determining the polymer properties of application importance (mechanical strength and hardness, surface morphology, wettability, electrical conductivity, dielectric properties and bioactivity) and those suitable for the characterization of the polymer structure and composition on microscopic scale, which in turn affect the macroscopic properties of polymers mentioned before. The latter techniques include, besides others, different sorts of optical and electron microscopies, X-ray photoelectron spectroscopy (XPS≡ESCA), X-ray and neutron diffraction, infrared and Raman spectroscopy (FTIR), secondary ions mass spectrometry (SIMS), atomic force microscopy (AFM) and several techniques based on elastic scattering of charged particles (RBS, ERDA, MEIS). In what follows selected techniques are briefly described with respect to their use for polymer characterization. Most of the diagnostic methods listed below are notoriously known and their principles are described in standard, easily available surveys [1]. For few techniques references to specialized publications are given.

Goniometry, measurement of surface wettability. Measurement of surface polarity and wettability (contact angle goniometry) is based on measurement of contact angle between a drop of a liquid and polymer surface. The contact angle is a direct measure of the polymer surface energy and consequently of the polymer wettability.

Ion beam analytical techniques [12]. Analytical techniques based on the use of energetic charged particles (protons, alpha particles, heavy ions) such as Rutherford Back Scattering (RBS), Elastic Recoil Detection Analysis (ERDA) and Nuclear Reaction Analysis (NRM) are very powerful because of broad spectrum of elements, which can be determined simultaneously with high sensitivity and excellent depth resolution down to few nanometers. The energy spectra of scattered or recoiled particles bring information on the sample composition and the depth structure of the sample as well. For all these methods the analysed depths are limited to several micrometers by the range of primary particles and typical detection limits vary from $10^{11}$-10$^{15}$ at.cm$^{-2}$. So that the time during which the samples are exposed to particle irradiation should be as short as possible and the radiation damages caused by analyzing beam should be taken into account when evaluating the experimental data. These methods are insensitive to chemical state of elements in the sample. General disadvantage of these methods is high cost of basic equipment, accelerator and data acquisition technique.

Neutron depth profiling technique (NDP) [13]. NDP is a special method for depth profiling of few light elements, namely He, Li, B and N in any solid material. The method makes use of specific nuclear reactions of these elements with thermal neutrons. The samples are placed in the neutron beam from nuclear reactor and the charged products of the neutron induced reactions (protons or alpha particles) are registered using a standard multichannel
spectrometer. From the measured energy spectra the depth profiles of above mentioned elements can be deduced by a simple computational procedure.

In X-ray Photoelectron Spectroscopy (XPS) the sample is irradiated by monochromatic soft X-rays which induce photoionization of the atoms and photoelectrons emitted from the sample surface layers, several nanometers thick, are analyzed by an electrostatic spectrometer. Photoelectron energy spectrum consists of peaks corresponding to electron energy states of the sample. By the XPS method it is possible to determine elemental composition of near surface layer of the sample and identify chemical state of the elements. Chemical state identification is based on measured energy shifts related to electron density in the atoms of interest. Apparent disadvantage of XPS method in some instances is too shallow sampling depth and rather high cost of instrumentation.

InfraRed Spectroscopy (IR). Infrared spectroscopy is an effective method for characterization of polymers as to chemical structure. IR spectra of the sample examined are obtained by two basic types of IR spectrometers: dispersive or Fourier transform (FTIR) ones. Infrared spectra are usually presented as a dependence of absorption (in percent transmission) on wave length or wave number. Any molecule or its part may be viewed as mechanical system exhibiting certain natural frequency at which it can be exited. The absorption bands in IR spectra correspond to these frequencies, which are characteristic for different chemical structures.

Raman Spectroscopy. Raman spectroscopy is an analytical technique that yields information about the molecular structure of sample materials based on observation of scattered light spectra. Small fraction of light scattered by molecules exhibits a frequency shift from the incident light which is related to the frequency of molecule vibrational states. It is similar to FTIR spectroscopy, but it has a few advantages, for example Raman can be used to study solids, liquids, powders, gels, slurries and aqueous solutions. Raman spectroscopy is based on detection of scattered light, i.e. the Raman effect. The radiation may be scattered elastically, that is without any change in its wavelength and this is known as Rayleigh scattering. Conversely the radiation may be scattered inelastically resulting in the Raman effect.

Both Raman and infrared spectroscopy provide qualitative and quantitative information about chemical species through the interaction of radiation with molecular vibrations. Raman spectroscopy complements infrared spectroscopy, particularly for the study of non-polar bonds and certain functional groups. It is often used as an additional technique for elucidating the molecular structure and symmetry of a compound. Raman spectroscopy also provides facile access to the low frequency region (less than 400 cm⁻¹ Raman shift), an area that is more difficult for infrared spectroscopy.

UV-Vis-NIR spectroscopy. UV-Vis spectroscopy probes the electronic transitions of molecules as they absorb light in the UV and visible regions. Any species with an extended system of alternating double and single bonds will absorb UV light and anything with with color absorbs visible light, making UV-Vis spectroscopy applicable to a wide range of materials. While UV-Vis and UV-Vis-NIR spectroscopy are well established analytical techniques being effectively applied across a broad spectrum of well-known industries, high performance UV-Vis spectroscopy has expanded into new applications, especially in the life sciences. Yet even in the traditional application areas such as materials characterization,
optics, coatings, glass, pharmaceutical and color control, UV-Vis spectroscopy has become more sophisticated, providing higher quality results faster and easier than ever. In the middle range of high performance instruments, you should expect UV-Vis coverage from 190 to 900 nm, and if NIR is needed, the range should extend from at least 190 to 3000 nm.

Measurement of electrical sheet resistance (two- or four-point method). The simplest technique for measurement of sheet resistance of polymer samples is two-point method, prerequisite of which is creation of two ohmic contact on the sample surface. The metallic contacts are commonly produced by sputtering or vacuum evaporation of suitable metal through a mask onto material surface. The conductivity measurement proceeds in standard manner.

Atomic Force Microscopy (AFM). The AFM provides quantitative morphological images of solid surfaces at a nanometer scale. It uses a sharp tip scanned over the sample surface to sense attractive or repulsive forces between the tip and the surface. The microscopic image of the surface is obtained as a surface, representing the locus of points of constant force between the tip and the sample.

The Scanning Electron Microscope (SEM) is a standard imaging technique based on electron back-scattering from the sample surface. It analyses the surfaces of solid objects, producing images with the resolution which is about order of magnitude better than that of optical microscopy (typically 10 nm). The SEM avoids the problem of thin samples (TEM) but the SEM observation requires the deposition of a thin conductive metal film on the sample surface to prevent sample charging.

Transmission Electron Microscopy (TEM) is a standard laboratory technique. TEM is an indispensable tool for high resolution observation of very fine structures on material surface. The resolution of TEM is about one order of magnitude better than that of SEM. It corresponds to 1 nm. There exist also high resolution transmission microscopes (HRTEM) with a resolution down to 0.1 nm, capable to resolve individual atomic lattice planes. Samples must be stable enough to withstand the electron beam impact during their examination. This can be a problem for polymers.

X-Ray Diffraction (XRD) is well known technique based on scattering of X-rays. It can be applied to semicrystalline or crystalline polymers and structural changes induced by modification. Occasionally observed shifts in the X-ray peak positions might indicate a distortion of the crystal structure due to increasing strain. The width of the X-ray reflection peaks yields information on the size of the crystals under investigation.

Electron Paramagnetic Resonance (EPR) is a technique used for detection and identification of unpaired free electrons (radicals) exhibiting spin magnetic moment. The method makes use of electron paramagnetic resonance corresponding to transition of electrons between states with different spin orientation initiated by absorption of rf electromagnetic signal in the sample. It is indispensable for detection of free radicals produced by modification of polymers.

Surface mechanical properties (nanoindentation). Nanoindentation technique has an important role in the characterisation of mechanical properties of materials on submicron scale. The ultra-low load indentation systems (nanoindentors) carry out continuous records of load and displacement as an indenter is pushed into a surface. Indentation curve can be used to determine mechanical properties even when the indents are too small to be imaged
conveniently. Analyse of this data gives a number of useful parameters and information for complete assessment of elastic, plastic and fracture properties of surface at the scale of real asperity contacts. The hardness, elastic modulus and other properties are evaluated from the indentation curve. The indentation methods can be used for the measurement of adhesion between the coating and the substrate when the indents create cracks and delamination on the coating or on the interface on polished section. The scratch test characterizes the surface mechanical properties of thin films and coatings, e.g. adhesion, fracture and deformation.

4. Evaluation of Cell-Material Interaction


This review is focused on the evaluation of the interaction between the artificial material and cells in cell culture conditions. In comparison with in vivo experiments, where both material and contacting cells are subjected to multiple and complex influences of the whole organism, the cell culture represents a relatively simple and well-defined system. This system enable screening of a wide range of materials and their surface modification in order to select the most appropriate variants for testing on laboratory animals and then for clinical studies. Therefore, the use of cell culture considerably saves these animals, and thus fulfills the ethical requirements for advanced scientific research.

Use of Cell Lines

In the first stage, the studies on cell-material interaction can be advantageously carried out on commercially available cell lines, including those derived from human patients undergoing surgery or from fetal tissues, such as human osteoblast-like MG-63 cells (European Collection of Cell Cultures, Salisbury, UK), obtained during osteosarcoma surgery in a 13-year-old boy, vascular smooth muscle cells of the line T/G HA-VSMC, derived from the human aorta, or human umbilical vein endothelial cells of the line HUVEC. The cell lines represent a relatively homogeneous and stable cell population, currently used for reproducible studies of the biocompatibility of various artificial materials, as well as for investigations of humoral factors influencing growth and differentiation of various cells. For these studies, it is necessary to select lines retaining markers characteristic for the identity, differentiation and maturation of a certain cell type, e.g., activity of alkaline phosphatase or production of osteocalcin typical for osteoblastic differentiation [14-17], presence of contractile proteins (alpha-actin, SM1 and SM2 myosins) in vascular smooth muscle cells or von Willebrand factor in endothelial cells [18-23].

Primocultures and Low-Passaged Cultures from Laboratory Animals

In the second stage of the in vitro studies on cell-material interaction, the selected results obtained on cell lines are verified on primary or low-passaged cultures obtained from the tissues of laboratory animals. For example, osteogenic cells can be isolated from the calvarias and long bones of newborn rats, e.g. by sequential collagenase digestion or the explantation
method [24-27]. Similarly, also vascular smooth muscle cells can be relatively easily isolated by the explantation method or enzymatical digestion from the rat aorta [18,19,21,23,28]. For isolation of vascular endothelial cells, vessels from larger animals, at least from rabbits or minipigs, are necessary in order to facilitate the manipulation with the tissue and to obtain a satisfactory cell yield. In order to avoid a potential contamination with smooth muscle cells, which are very vital and prone to overgrow the endothelial cells, the best approach seem to be to fill the vessel with collagenase diluted in cell culture media and incubate the vessel in a cell incubator without any scraping of endothelial cell layer. The released endothelial cells are then collected by centrifugation ad seeded into small amount of culture media of appropriate chemical composition, e.g. a higher (20%) content of fetal bovine serum, presence of glutamine, pyruvate, non-essential amino acids and other specific supplements [29-31]. The artificial materials material can be also tested with stem cells, e.g. those obtained from bone marrow, in order to set appropriate physical and chemical properties of the developed artificial material, which would promote the differentiation of stem cells into a desired cell type [32].

**Primocultures and Low-Passaged Cultures from Human Patients**

In the third stage of the cell-material interaction studies, the selected results can be further verified in primary or low-passaged cultures obtained from human patients undergoing surgery or biopsy. Such cells are also commercially available from specialized companies (e.g., Provitro GmbH, Berlin, Germany; http://www.provitro.de), thus scientific workers from non-clinical institutions have possibility to avoid potential difficulties associated with obtaining the ethical approval, which is usually required for the experimental use of human cells and tissues.

**Static and Dynamic Cell Culture Systems**

The cultivation of cells obtained from all mentioned sources on the investigated materials usually starts in a conventional static cell culture system. The material samples are inserted in plastic dishes or bottles, immersed with cell culture media, containing defined concentrations of suspended cells, and incubated at 37°C in a humidified air atmosphere with 5% of CO₂ [33]. However, the relatively simple and highly reproducible manipulation with cells and materials in the static cell culture system is compromised by a relatively low supply of oxygen and nutrients to cells, as well as relatively slow waste removal, especially if the investigated artificial material is constructed in a so-called three-dimensional form, i.e. as scaffolds enabling ingrowth of cells inside the material. For these advanced tissue engineering constructs, a more appropriate environment is represented by dynamic cell culture systems, e.g. perfusion systems (http://www.provitro.de) or rotary bioreactors (http://www.cellon.lu). These systems enable the flow or penetration of fresh cell culture media through the material. In addition, these systems can provide defined mechanical stimulation of cells, so that they better resemble the physiological environment of cells in situ conditions and accelerate the maturation and establishment of specialized function of a given cell type. The dynamic cell culture systems are necessary especially for the construction of bioartificial vascular prostheses, i.e. synthetic grafts with reconstructed tunica intima containing endothelial cells or even tunica media with smooth muscle cells [30,34-36], as
well as for the creation of bioartificial bone tissue on the basis of three-dimensional porous scaffolds [37].

4.2. Specific Markers of Cell Behavior in Cell-Material System in Vitro

During years of studies on cell-material interaction, we have developed a complex test of basic and more specialized markers of cell adhesion, growth, differentiation, viability/cellular damage, stress adaptation, immune activation as well as potential mutagenicity and cancerogenicity of the material. These markers can be investigated by a wide range of microscopical and biochemical methods, such as conventional optical, confocal and scanning electron microscopy, immunofluorescence and immunoperoxidase staining, enzyme-linked immunosorbent assay (ELISA), flow-cytometry, electrophoresis, immunoblotting, immunoprecipitation, microarray techniques or cell transfection, depending on the facilities in a given scientific laboratory.

**Table 1. Examples of specific amino acid sequences in extracellular matrix molecules, which represent ligands for cell adhesion receptors and can be used in tissue engineering.**

<table>
<thead>
<tr>
<th>Amino acid sequence</th>
<th>Typical for a certain ECM molecule</th>
<th>Preferred by a certain cell adhesion receptor</th>
<th>Preferred by a certain cell type</th>
</tr>
</thead>
<tbody>
<tr>
<td>RGD*</td>
<td>Vitronectin, Fibronectin</td>
<td>Integrin $\alpha_1\beta_3$#</td>
<td>Without significant preference</td>
</tr>
<tr>
<td>DGEA</td>
<td>Collagen</td>
<td>Integrin $\alpha_5\beta_1$</td>
<td>Without significant preference</td>
</tr>
<tr>
<td>KQAGDV</td>
<td>Vitronectin, fibronectin</td>
<td>Integrin $\alpha_1\beta_3$#</td>
<td>Vascular smooth muscle cells</td>
</tr>
<tr>
<td>VAPG</td>
<td>Elastin</td>
<td>Non-integrin receptor</td>
<td>Vascular smooth muscle cells</td>
</tr>
<tr>
<td>REDV</td>
<td>Fibronectin</td>
<td>Integrin $\alpha_4\beta_1$</td>
<td>Vascular endothelial cells</td>
</tr>
<tr>
<td>YIGSR, IKVAV</td>
<td>Laminin</td>
<td>Integrins $\alpha_6\beta_1$, $\alpha_7\beta_1$</td>
<td>Neurons</td>
</tr>
<tr>
<td>KRSR</td>
<td>Part of the heparin binding domain</td>
<td>Non-integrin receptor</td>
<td>Osteoblasts</td>
</tr>
</tbody>
</table>

*The possibility of binding RGD to different receptors is probably caused by a different spatial conformation of RGD due to the vicinity of different amino acids or existence of different synergistic sequences on various extracellular matrix (ECM) molecules. # At the same time, one type of integrin can bind more ligands [38-40].
Indicators of Cell Adhesion

Protein adsorption. The cell adhesion on artificial materials is mediated by adsorption of extracellular matrix (ECM) molecules or molecules participating in tissue regeneration. These molecules, represented e.g. by vitronectin, fibronectin, collagen, laminin, fibrinogen and fibrin, can be spontaneously adsorbed to the material surface from the serum supplement of the culture media in vitro or blood and other body fluids in vivo. In addition, many cell types are able to synthesize and secrete these molecules, and to deposit them on the material surface. Even after trypsinization of cells (in order to detach them from the original cell culture flask or dish and seed them on the tested material), the cells usually contain a considerable amount of these molecules on their membrane. After adsorption of cell adhesion-mediating molecules, the cells bind active sites on these molecules, represented by a wide range of specific amino acid sequences, such as Arg-Gly-Asp (RGD), Asp-Gly-Glu-Ala (DGEA), Lys-Gln-Ala-Gly-Asp-Val (KQAGDV), Val-Ala-Pro-Gly (VAPG), Arg-Glu-Asp-Val (REDV), Lys-Arg-Ser-Arg (KRSR), Ile-Lys-Val-Ala-Val (IKVAV) or Tyr-Ile-Gly-Ser-Arg (YIGSR) (Table 1, Figure 4). These sequences are usually typical for a certain ECM molecule. Also they are usually preferred by a certain cell adhesion receptor as well as by a certain cell type (Table 1) [38-41], which can be advantageously used in tissue engineering, as described below. In addition, these adhesion motifs cooperate with other synergistic amino acid sequences, e.g. PHSRN, which help to maintain appropriate spatial conformation of these ligands as well as integrin receptors [40-42].

Figure 4. Principle of the cell adhesion to artificial materials. In cell culture media or body fluids, the material is spontaneously adsorbed with cell adhesion-mediating extracellular matrix proteins (e.g., vitronectin, fibronectin). The cells then adhere to specific amino acid sequences of these proteins by their adhesion receptors of integrin or non-integrin type [38-41].

As evident from the text mentioned above, for appropriate cell adhesion, the cell adhesion-mediating molecules should be adsorbed not only in a sufficient amount and homogeneity, but
also in an appropriate spatial conformation enabling a good accessibility of active sites on these molecules by adhesion receptors on the cell membrane (Figures 4-6). If the primary spatial conformation of the adsorbed proteins is not suitable for their binding to cell adhesion receptors, the cells can actively reorganize it, if the proteins are adsorbed in a flexible form, which usually happens on wettable surfaces. On the contrary, on highly hydrophobic surfaces (water drop contact angle about 100°), the cell adhesion mediating molecules are usually too rigid, and thus the cell adhesion is often insufficient, although the absolute amount of the adsorbed molecules can be even higher than on wettable surfaces. In addition, highly hydrophobic surfaces are known to promote the preferential adsorption of albumin, which is non-adhesive for cells [18,19,40,41,44-48].

![Fluorescence of collagen IV conjugated with Oregon Green 488](image)

*Figure 5. Fluorescence of collagen IV conjugated with Oregon Green 488 (Molecular Probes, Eugene, OR, USA.) spontaneously adsorbed to unmodified polyethylene foils (A) or polyethylene modified with 1014 O+ ions/cm² at the energy of 30 keV (B). Collagen was diluted in phosphate-buffered saline to the concentration of 0.02 mg/ml (10 µg/cm²) and incubated with the foils for 24 h at room temperature. For autofluorescence control, unmodified (C) and ion-irradiated foils (D) were not exposed to the collagen solution. Microscope Nikon, CCD camera, Image Pro Plus 3.0 software [43].*
The type and amount of proteins adsorbed to the biomaterial surface can be determined by immunocytochemical studies using specific antibodies. Also commercially available proteins conjugated with fluorescence markers can be used for quantification of protein adsorption as well as for the evaluation of the homogeneity of the protein distribution on the material surface (Figures 5, 6). However, the studies on the spatial conformation of the adsorbed ECM molecules are more difficult and are still in their beginning. This problem can be partially solved by some physicochemical methods originally developed for the investigation of artificial materials, e.g., observation of the surface morphology of the adsorbed protein layer by scanning electron microscopy (SEM) or atomic force microscopy (AFM), studies of the protein layer using surface plasmon resonance (SPR), mass spectrometry (e.g., MALDI-TOF), determination of the chemical composition of the protein layer by various spectroscopic methods, like Fourier transform infrared spectroscopy (FTIR), X-ray photoelectron spectroscopy (XPS), Auger spectroscopy (AES) or Raman spectroscopy, or by indirect immunocytochemical studies using antibodies against specific regions on ECM molecules or certain types of adhesion receptors preferentially expressed on adhering cells in response to the composition of the surface of the adsorbed protein layer [16,31,44-46,48-51].

Expression of cell adhesion receptors. Proteins adsorbed on the biomaterial surface are bound by adhesion receptors on the cell membrane. The most known and most systematically described adhesion receptors are integrins. Integrins are heterodimeric transmembrane glycoproteins consisting from one alpha and one beta chains. Their function is dependent on calcium, which binds on the alpha subunit. About 16 different subunits alpha and 8 subunits beta were described. However, these numbers are not definitive, because integrin receptors are still under intensive research and new subunits are being revealed. Various combinations of alpha and beta chains result in constitution of receptors with preferential affinity to certain ECM molecules. In addition, certain integrins are present preferentially on certain cell types.
Thus, these cell types adhere preferentially to certain amino acid sequences and ECM proteins (Table 1) [38-40,52,53]. Recent investigations have shown that other receptors than integrins can also take place in cell-matrix adhesion, e.g. proteoglycan-based receptors. These non-integrin receptors bind not only aminoacid sequences on ECM molecules but also saccharide-based parts of these molecules. For example, heparan sulphate proteoglycan on osteoblasts recognizes a bone-specific oligopeptide KRSP, or an asialoglycoprotein receptor on hepatocytes binds a galactose ligand. Also the VAPG motif on elastin is recognized by a non-integrin receptor, represented by a multifunctional 67-KDa peripheral membrane protein [38-40,52,53].

Assembly of focal adhesion plaques. It was well described in integrin receptors that after ligand binding, these receptors are recruited into distinct dot-like or streak-like nano- or microdomains on cell membrane, called “focal adhesion sites”, “focal adhesion plaques” or simply “focal adhesions” (Figure 7). In these regions, the integrins communicate with many specific structural and signaling molecules. The former are represented by proteins of membrane-associated cytoskeleton, also called “focal adhesion proteins”, such as talin, α-actinin, filamin, paxillin or vinculin. These proteins act as linkers between the integrin receptors and the cytoplasmatic actin cytoskeleton, which is associated with nuclear membrane, membranes of cellular organelles as well as with various enzymes, and thus influences intracellular processes important for cell behavior, including transport and secretion of various molecules, endocytosis, decision between cell proliferation and differentiation or apoptosis (Table 2). The signaling molecules are represented e.g. by cytohesin-1, focal adhesion kinase (FAK), integrin-linked kinase (ILK), mitogen-activated protein kinases (MAPK), extracellular signal-regulated kinase (ERK), β3-endonexin, integrin cytoplasmic-domain-associated protein-1 (ICAP-1), receptor for activated protein kinase C (Rack-1), calcium- and integrin-binding protein (CIB) or small GTPases.

Figure 7. Talin-containing streak-like focal adhesion plaques (A) and beta-actin cytoskeleton (B) in vascular smooth muscle cells in cultures derived from the rat aorta. Immunofluorescence staining, the nuclei counterstained with propidium iodide (A) or Hoechst 33342 (B). Microscope Olympus IX 50, digital camera DP 70, obj. 100, bar=20 µm.
Both structural and signaling molecules play a decisive role for further behavior of cells after contacting a biomaterial, i.e., switching between cell proliferation and differentiation, survival or apoptosis and other functions [38-40,52,53].

Number, spreading and shape of adhering cells. The engagement of cell adhesion receptors and formation of focal adhesion plaques are determining factors for the number of initially attached cells as well as the size and shape of their spreading area. The number and spreading area can be used as relatively simple but accurate and important criteria for evaluation of initial cell adhesion as well as the cell behavior in late culture intervals. The cell number can be determined by counting cells directly on the material in a microscope. On transparent materials, living cells in native cultures can be evaluated. On non-transparent materials, both living and fixed cells can be visualized by various fluorescence methods including transfection of cells with fluorescence proteins or scanning electron microscopy [10,14,16,18,19,21,23,41,54]. The microscopic methods are also suitable for measurement of the size of cell adhesion area, i.e. the cell area projected on the material surface, which can be measured on digital pictures using an image analysis software [16,31,41,54]. For cell counting, the cells can be also detached from the material surface by proteolytic enzymes (usually trypsin) and/or ethylene-diamino-tetraacetic acid (EDTA), a potent calcium chelator. The cells in suspension then can be counted manually in a hemocytometer or automatically in various cell counters or cell viability analysers, i.e. instruments in which cells are stained with trypan blue. This stain penetrates the membrane of dead cells, whereas the live cells

<table>
<thead>
<tr>
<th>Cell behavior/Cell spreading</th>
<th>Viability</th>
<th>Migration</th>
<th>Proliferation</th>
<th>Differentiation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Small</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>Medium</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↓</td>
</tr>
<tr>
<td>High</td>
<td>↑</td>
<td>↓</td>
<td>↓</td>
<td>↑</td>
</tr>
</tbody>
</table>
remain unstained. In addition, the resistance of cells to the detachment by proteolytic enzymes can serve as an important indicator of the strength of cell adhesion [18,19,21,23].

The cell number is often estimated indirectly by evaluation of the cell metabolic activity using tests of activity various enzymes, e.g. esterases or mitochondrial enzymes (MTT, MTS or XTT tests), or measurement of DNA content in cell lysates [11,37,43,55,56]. The indirect methods are suitable particularly for cells growing inside three-dimensional materials, not accessible for direct cell counting.

**Indicators of Cell Growth**

Growth of cells comprises their hyperplasia (proliferation), i.e. increase in cell number, and hypertrophy, manifested by the increase in cell volume and protein content, which is often associated with the increase in the DNA content and chromosome number, resulting in polyploidy. Polyploidy is a well known feature e.g. in vascular smooth muscle cells, where it is considered as an adaptation of cells to increased mechanical or hormonal stimulation [21].

The cell proliferation can be evaluated by counting cells on the tested material in several time intervals, followed by the constructing growth curves and calculating cell population doubling time. Doubling time (DT) can be calculated according the following formula:

$$DT = (t-t_0) \log 2 / \log N_t - \log N_{t_0},$$

where \(t_0\) and \(t\) represent earlier and later time intervals after seeding, respectively, and \(N_{t_0}\) and \(N_t\) represent the number of cells at these intervals [7,16,18,19,21,23].

Important markers of cell proliferation are also represented by various parameters of the cell cycle, such as the duration of the whole cell cycle and its phases, distribution of cells within the phases of the cell cycle, distribution of DNA content, incorporation of bromodeoxyuridine (BrdU) into newly synthesized DNA (i.e., a parameter showing the S-phase fraction, i.e., the percentage of cells actually synthesizing DNA), expression of proliferation antigens, such as PCNA or Ki 67 (the presence of Ki-67 antigen reveals the growth fraction, i.e., the percentage of cells capable of entering the mitotic cycle). The cell cycle parameters can be advantageously evaluated by immunocytochemical and flow-cytophotometric approaches [18,21,23,57]; an older but accurate approach is represented by \(^{3}\)H-thymidine autoradiography [58,59]. Also the presence and activity of various enzymes taking part in the cell cycle, such as cyclin-dependent kinase 2 (CDK2) and its endogenous activators and inhibitors can serve as precise indicators of the cell proliferation capacity. CDK2 is a key stimulator of cell cycle progression, acting in G1/S, S-phase and G2/M boundary of the cell cycle. Its activity is supported by both cyclin A and E and inhibited by proteins p21 and p27 [60-62].

The cell volume can be calculated from cell diameters measured either manually in cells in suspension on microscopic slides or automatically in a flow cytometer. Also certain types of cell counters provide automated information on cell diameter [14,18,19,21,23].

The protein content is measured by biochemical methods, such as methods of Lowry or Bradford [14,18,19,21,23], now usually by commercially available kits. The DNA content can be measured by flow cytometry, and the chromosomes can be counted directly in cells after their arrest in the metaphase of the mitosis by Colcemide [21].
**Indicators of Cell Differentiation**

Recently it appears that the cell differentiation can be essentially influenced not only by the presence of various humoral factors in the surrounding environment, but also by physical and chemical properties of the artificial growth support. For example, human mesenchymal stem cells in cultures on soft polyacrylamide matrices that mimicked brain differentiated towards neurons, whereas stiffer matrices mimicking muscle were myogenic, and comparatively rigid matrices that mimicked collagenous bone proved osteogenic [32]. As indicators of cell differentiation, the presence, content and spatial distribution of molecules specific or typical for certain cell types are usually followed. For example, typical markers of VSMC differentiation are represented by a higher content of contractile proteins alpha-actin and SM-myosins, and their assembly into filaments (Figure 8). Other markers of VSMC differentiation are desmin, caldesmon, calponin and meta-vinculin [18-23,40,41]. Mature vascular endothelial cells are characterized by a high content of von Willebrand factor stored in Weibel-Palade bodies, presence of CD 31 and CD 34 antigens, as well as the formation of thick beta-actin cables [19,31,34,63]. As markers of osteogenic differentiation, high activity of alkaline phosphatase, production of collagen I and non-collagenous calcium-binding ECM glycoproteins osteocalcin and osteopontin are currently evaluated [14-17,24-27,37,47].

**Indicators of Cell Viability, Stress Adaptation and Death**

Good adhesion, spreading, assembly of focal adhesion plaques and cytoskeleton as well as a high proliferation and differentiation activity mentioned above are also signs of good cell viability and non-cytotoxicity of the tested material. In addition, the above mentioned tests of the activity of mitochondrial enzymes, such as MTT, MTS or XTT, often used for evaluation of cell growth, can be considered as tests of cell viability and metabolic activity rather than tests of cell proliferation.

![Figure 8. Concentration of markers of adhesion (A) and differentiation (B) in rat aortic smooth muscle cells in cultures on polyethylene (PE) modified by irradiation with O⁺ ions (energy 30 keV, doses from 10¹³ to 10¹⁵ ions/cm²). Measured by enzymatic immunosorbent assay (ELISA) per mg of protein, absorbances expressed in % of the values obtained on pristine non-modified PE. Mean ± SEM from 4 experiments, Student t-test for unpaired data, *: p<0.05 **: p<0.01 compared to the values on pristine PE.](image-url)
Sometimes the cells can be highly metabolically active without a considerable proliferation [10,37,43,55]. Long-term tolerance of the newly developed layers by cells can be assessed by cultivating cells on these materials for a period of several months [18].

Stress adaptation of cell growing on artificial materials can be estimated by the presence and content of heat-shock proteins, such as HSP 60 and HSP 70, and other chaperone molecules [64]. For detection of dead cells, staining of dead cells with trypan blue, propidium iodide and chromatin dye Hoechst 33258 can be applied (the latter visualizes chromatin clumps in cell nuclei typical for apoptosis; [65]). These dyes penetrate through damaged cell membrane, whereas viable cells with intact membrane remain unstained. Simple fluorescence staining is often a principle of various commercially available kits developed for quick evaluation of potential material cytotoxicity. For example, in a commercially available LIVE/DEAD viability/cytotoxicity kit (Invitrogen), the cells are incubated in two probes, i.e. calcein AM producing green fluorescence and detecting the esterase activity in living cells, and ethidium homodimer-1 emitting red fluorescence and signalizing the membrane damage in dead cells. For deeper studies on cell death (necrosis and apoptosis), a wide range of more sophisticated approaches are available, e.g., studies on annexin V, caspase activity, p53 expression or DNA fragmentation [66,67]. However, the studies on cell death represent a large independent scientific field exceeding the frame of this review.

**Indicators of Potential Mutagenicity, Carcinogenicity and Immunogenicity of the Material**

Some new materials perspective for advanced biomedical technologies, especially carbon nanoparticles like fullerenes, are potentially mutagenic, carcinogenic and immunogenic [16,65]. Therefore, standard tests of the morphological transformation of Syrian hamster embryonic cells in cultures on these materials (described in detail by [68,69]) can be performed. Immune activation of bone and vascular cells on the materials can be estimated by increased concentration of immunoglobulin and selectin adhesion molecules (ICAM-1, VCAM-1, ELAM-1), which bind cells of the immune system [15,16,18,19,23], as well as by the production of cytokines, such as tumor necrosis factor alpha or interleukins beta [55].

**5. Reactions between Plasmas and Polymer Surface**

**Surface Chemistry**

The characterization of the surface chemistry of the modified polymer is one step in understanding the mechanism for cells adhesion. The next crucial step is to determine the nature and extent of chemical interactions between the overlayer of interest and the modified polymer surface. This step presents a challenge because currently there are no techniques available with the sensitivity to characterize chemical interactions for an atomically thin buried interface. Several approaches have been used to analyze buried interfaces. Ion sputter depth profiling (typically done with Ar ions) in conjunction with XPS can be used to evaluate a buried interface for overlayers >10 nm [2].
The parameters of treatment were chosen since these led to the most pronounced changes of polymer surface in our previous experiments [70-74]. It was observed elsewhere that plasma treatment of polymer macromolecules results in their cleavage, ablation, alterations of chemical structure and thus affects surface properties e.g. solubility [75]. The chemical structure of modified polyethylene (PE) was characterized by FTIR and XPS spectroscopy. Exposition to discharge leads to cleavage of polymeric chains and C–H bonds followed by generation of free radicals which easily oxidize [10,76]. By FTIR spectroscopy the presence of new oxidized structures within whole specimen volume can be detected. IR spectra in the 1710–1745 cm\(^{-1}\) interval [71,77] from PE, exposed to Ar plasma, show the presence of the oxidized structures (carbonyl, carboxyl, ester).

The chemical structure of plasma-treated PE was examined using XPS, the oxygen concentration profile was determined from RBS measurement and the concentration of free radicals was determined by EPR technique [78]. The -CH\(_2\), -CH\(_3\) (of pristine PE), oxidized (-\(\text{C}=\text{O}\), -\(\text{COO}\), -\(\text{COC}\)), -\(\text{NH}_2\), and also -\(\text{C}=\text{C}\) groups (typical for aromates) were reported present at Ar plasma-treated high-density PE (HDPE) [70].

Depth profile and total content of oxygen in PE was determined by RBS method. The profiles of plasma-treated sample and that subsequently etched in water are compared in Figure 9. The mean oxygen contents in the surface layer accessible by RBS, ca 140 nm thick, are \((39\pm5)\) and \((28\pm3)\) \(10^{15}\) cm\(^{-2}\) for plasma-treated and water etched samples respectively. Both concentration profiles exhibit a maximum at the depth of ca 20 nm, further the concentration decreases slowly, falling to negligible concentrations at 60 nm. Similar trend was observed in previous report dealing with PE exposed to Ar plasma at the power of 1.7 W [70].
Due to plasma treatment the radicals (R, free spin) are generated on polymer chain. Not only C-H but also C-C bonds are likely to brake, the later leading to fragmentation of polymer chain. The radical concentration determined by EPR method is in Table 3. The concentration of free radicals R decreases during the aging to about quarter of the initial after 80 days. The decrease of R is a result of radical recombination [h]. Detailed comment of EPR observation („aging“ of radicals) is described too [79].

The oxygen concentration in the first 10 monolayers of modified PE was followed independently by XPS (see Table 3) [80]. The oxygen concentration was determined from survey spectra shown in Figure 10. It is confirmed that plasma treatment leads to oxidation of the surface layer and the oxygen concentration is 31 at.% 1 hour after plasma treatment. One can see from Table 3 that with increasing aging time the oxygen concentration decreases due to rearrangement of the treated polymer chains and their fragments [74,81]. The profile and area of individual peak in XPS spectra enable to determine chemical group concentration in PE surface (see Table 4). Model deconvoluted C(1s) peak is shown in Figure 11. In Table 4, the concentration of detected groups -CH₂-, -C-O-, -C=O and -O-C=O is presented [78,82]. One can see that during sample aging the concentration of -C=O group increases and -O-C=O decreases. Only a trace concentration of nitrogen was detected.

Above described results prove that plasma treatment of PE leads to cleavage of molecular chain and production of polar groups. Since these changes may enhance interaction with polar solvents, the water etching of modified PE was examined; the calculated thickness of
the surface layer removed from plasma-modified PE (240 s, 8.3 W) by 24 hour water etching is 20.6±4.6 nm [78].

Table 3.C (1s) a O (1s) concentrations (at. %) determined from XPS measurements and radical densities (R, 10¹⁸ g⁻¹) determined by EPR method. The concentrations and densities were determined on the pristine PE, plasma-treated PE (1 or 24 hours after the treatment) and plasma-treated and water etched PE (see left hand side of the table). Before the measurement the samples were stored under standard laboratory conditions [78].

<table>
<thead>
<tr>
<th>PE modification</th>
<th>C</th>
<th>O</th>
<th>Radical density</th>
</tr>
</thead>
<tbody>
<tr>
<td>pristine PE</td>
<td>100</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>1 h after plasma treatment</td>
<td>69.2</td>
<td>30.8</td>
<td>-</td>
</tr>
<tr>
<td>24 h after plasma treatment</td>
<td>75.7</td>
<td>24.3</td>
<td>2.77</td>
</tr>
<tr>
<td>plasma treatment and 24 h in water</td>
<td>63.4</td>
<td>36.6</td>
<td>2.31</td>
</tr>
</tbody>
</table>

Figure 10. The XPS survey spectra of PE modified by plasma (plasma/PE) or modified by plasma and water etched (H₂O/plasma/PE) measured 24 h after the plasma treatment. Before the measurement the samples were stored under standard laboratory conditions [78].

Chemical composition of the dissolved material was determined by FTIR spectroscopy. FTIR spectrum of the sample obtained as a deposit after evaporation of water is shown in Figure 12. The etched material contains -CH and -CH₂ groups of original PE molecular chain. Furthermore the nitrogen (-NH, -N-O) and oxygen containing (-C=O, -COOC-, C-O-) groups were detected. These were introduced either by the residual reactor atmosphere or during the
post treatment storage. The polar groups are responsible for enhanced water solubility of the modified PE [78].

Table 4. Result of evaluation of XPS C(1s) peak for the PE samples 1 and 24 hours after the plasma treatment and plasma treated PE sample subsequently water etched for 24 hours. Concentrations of selected groups are in at. % [78]

<table>
<thead>
<tr>
<th>Chemical group</th>
<th>PE modification</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 h after plasma</td>
</tr>
<tr>
<td>-CH₂-</td>
<td>65.8</td>
</tr>
<tr>
<td>-C-O-</td>
<td>26.1</td>
</tr>
<tr>
<td>-C=O</td>
<td>1.6</td>
</tr>
<tr>
<td>-O-C=O</td>
<td>6.5</td>
</tr>
</tbody>
</table>

Figure 11. C(1s) XPS spectrum of PE measured 1 h after the plasma treatment (240 s, 8.3 W). The dashed line represents the measured intensities. [78].
Ablation and Depth of Modification

In general, the depth of surface modification mainly depends on the power of the plasma discharge and treatment time [83]. For plasma-treated polymer samples, the depth of the surface modification is typically of several tens nm and has been studied by surface-sensitive techniques such as angle-resolved XPS [n] or RBS [70,78]. In [70,78] the concentration depth profile of oxygen, incorporated in the polymer surface layer (high- and low-density PE, HDPE and LDPE) as a result of the plasma treatment, was determined (Figure 13) [70]. With increasing exposure time to plasma discharge oxygen amount in polymer surface layer also increases in both, LDPE and HDPE. The deeper penetration of oxygen up to about 120 nm is observed in HDPE plasma-treated for 25 s. After treatment for 250 s, however, most of oxygen is incorporated in surface layer only about 50 nm thick. The evolution of the oxygen concentration depth profiles in LDPE is similar.

It was observed elsewhere that plasma treatment of polymer macromolecules results in their cleavage, ablation, alterations of chemical structure and thus affects surface properties e.g. solubility [75]. The effects of the treatment in Ar plasma on the surface properties of PE were examined in [72]. The parameters of the plasma discharge (240 s, 8.3 W power) were chosen, on the basis of our previous experiments [70-74,78], to guarantee the most pronounced changes of polymer surface. Mean thickness of the ablated PE layer was calculated from the weight difference measured by gravimetry. By Ar plasma (8.3 W, 240 s) 30±8 nm thick surface layer is ablated and thickness of the surface layer removed from plasma-modified PE by 24 hour water etching is 21±5 nm [78]. The surface morphology of PE was examined by AFM method (Figure 14, [78]). Obviously the exposition to plasma discharge leads to dramatic topology change. As ablation result a lamellar structure on the sample surface appears [70]. It can be concluded, with regard to the earlier data [84], that PE
amorphous phase is ablated faster than the crystalline one. Surface morphology of this sample after subsequent water-etching is shown in Figure 14.

Figure 13. Concentration depth profile of oxygen incorporated in HDPE and LDPE modified in the Ar plasma at the discharge power of 1.7 W. The numbers are the times of plasma treatment in seconds. The profiles were determined by RBS technique [70].

Figure 14. AFM images of pristine, plasma-modified and plasma-modified and water-etched PE. $R_s$ is the surface roughness in nm [78].
It is seen that water etching removes tiny, sharp formations from the sample surface and the etched surface exhibits lower diversity but higher surface roughness $R_a$. In analogy with earlier reports [75,85] obtained on LDPE and polypropylene (PP) plasma-modified samples, it can be concluded that the observed changes correspond to removal of low-molecular oxidized segments from plasma-modified polymer surface [72].

**Surface Properties**

**Surface Morphology**

In most of published studies of polymer modification by plasma treatment, great attention is devoted to the roughness and surface morphology of modified polymers e.g. reviews [1,2]. Exposure to plasma discharge results in significant changes of surface morphology of high- and low-density polyethylene (HDPE and LDPE) which are seen from SEM images of pristine and plasma-treated polymers [70]. While on the surface of plasma-modified HDPE a lamellar structure of macromolecular chains is visible, on the surface of modified LDPE formations are created which may reflect spherulitic arrangement of macromolecular chains [70]. AFM examination of the same samples was accomplished to verify the SEM observation. The AFM results for selected samples are shown in Figure 15. Surface roughness is an increasing function of the scan dimension and plasma exposure [70]. Analogous results were reported in [86]. Plasma treatment results in a mild increase of the surface roughness for LDPE but in much stronger increase for HDPE.

![Figure 15. Contact mode AFM height images of PE foils before (HDPE and LDPE) and after 400 s modification in Ar plasma (HDPE/400, LDPE/400). [70].](image-url)
Surface Wettability and Aging Effects (Stability)

Long-term stability of a modified polymer surface is important for their potential practical applications. Adverse aging effects may be significant if the polymer is not stored in a controlled environment or coated immediately after treatment. The stability of a modified surface depends on several factors including chemical structure of the modified polymer which in turn depends on the polymer initial structure and composition, plasma treatment procedure (working gas, discharge power) and the environment in which the polymer is stored. It has been observed by many researchers that plasma-modified polymer surfaces are highly susceptible to aging effects [1,2,83,87-92].

It is known that the value of the contact angle (i.e. polarity and wettability) is mostly affected by the chemical structure and morphology of the polymer surface layer [93,94]. In Figure 16, the dependence of the contact angle on the exposure time to Ar plasma at 8.3 W discharge power is shown [71].

![Figure 16. Dependence of the PE contact angle on the exposure time to 8.3 W plasma discharge. The contact angle was measured at different times elapsed from the plasma treatment. The numbers are the times in hours elapsed from the plasma treatment [71].](image-url)
With increasing exposure time the contact angle decreases. It was found that the enhanced wettability (low contact angle) of the plasma-treated PE is not permanent and it declines with the time elapsed from the plasma treatment. With increasing time from the plasma exposure the contact angle increases and the increase is more pronounced for the specimens exposed to plasma for longer time. The increase is so strong that after 120 h of ageing the contact angle of the PE sample, plasma-treated for 240 s, becomes larger than that of pristine PE. It was found that the contact angle gradual increase practically stops after 336 h, i.e. 14 days after exposition to plasma. Relaxation of the surface hydrophilicity is obviously connected with a rearrangement of degraded macromolecules on the surface of modified PE [84]. An inward diffusion of low mass oxidized fragments and an orientation of polar groups toward specimen bulk are also supposed [89].

Using angle resolved XPS (ARXPS) technique, the chemical structure of the first ca 10 monolayers (a surface layer about 5 nm thick) of the sample surface can be analyzed [74]. For plasma-treated samples, the oxygen concentration was followed as a function of the aging time of the samples. The oxygen concentrations were determined from ARXPS spectra using standard procedure [82]. In the first monolayer of pristine PET the oxygen concentration is below the value expected from the PET stoichiometry, the deficit could indicate the preferential orientation of the oxygen containing groups inwards resulting from the process of fabrication of oriented PET foil. Immediately after the plasma treatment the oxygen concentration increases, but then it decreases with increasing aging time. These results are in agreement with the results of the contact angle measurements [74], the evolution of which is explained by the rearrangement of degraded macromolecules and molecular fragments.

The oxygen concentration in the first 10 monolayers of plasma-modified PE was followed independently by XPS (see Table 3) [78]. The oxygen concentration was determined from survey spectra shown in Figure 10. It is confirmed that plasma treatment leads to oxidation of the surface layer and the oxygen concentration is 31 at.% 1 hour after plasma treatment. One can see from Table 3 that with increasing aging time the oxygen concentration decreases due to rearrangement of the treated polymer chains and their fragments [78,81]. The profile and area of individual peak in XPS spectra enable to determine chemical group concentration in PE surface (see Table 4). Model deconvoluted C(1s) peak is shown in Figure 11. In Table 4 concentration of detected groups -CH2-, -C=O, -C-O and -O-C=O is presented [82]. One can see that during sample aging the concentration of -C=O group increases and -O-C=O decreases. Only trace concentration of nitrogen was detected.

**Mechanical Properties**

Mechanical properties of polymer surface layer are affected by changes in chemical structure due to exposure to plasma discharge [95]. Same mechanical properties of pristine PE and PE modified in Ar plasma discharge were studied using NanoIndenter XP MTS device [71]. The scratch results for pristine PE and PE plasma-modified for time 240 s were chosen. No difference between scratch characteristic of pristine and modified PE is observed. Also there are no signs of plastic deformation and only mild mechanical damage of surface layer is seen. It may be therefore concluded that the deformation of the PE surface layer is
mostly elastic. Elastic modulus and hardness as a function of the exposure time for pristine and plasma-modified PE are shown in Figure 17. It is seen that the modulus (Figure 17A) increases as a result of the plasma treatment. For 240 s exposition the modulus achieves its maximum. Dependence of the PE hardness on the exposure time and displacement into surface is shown in Figure 17B. Plasma treatment for short times does not change, within experimental errors, the PE hardness. Significant hardness increase observed on the PE sample treated for 240 s may be due to polymer crosslinking initiated by plasma discharge [95].

Figure 17. Dependence of the elastic modulus (A) and hardness (B) on the displacement into surface measured on pristine PE and PE exposed to Ar plasma for 10, 30 and 240 s [71].

6. Modification of Polymers by Ion Implantation

Compositional and Structural Changes of Polymers under Low-to Medium-Energy Ion Implantation

The ion implantation is a well-established technique for modification of properties of solids. It has been used for modification of polymers since its invention on the beginning of 60-ties [3,4,96]. Huge number of experimental results has been obtained on interaction of low-medium energy ions with polymers on a microscopic level and on structural and compositional changes of polymers resulting from ion irradiation. The effect of ion irradiation on polymer structure and composition is a result of complex physical and chemical processes initiated by energy deposited by ion in polymer matrix. Since the energy
of bond dissociation in polymers does not exceeds 10 eV, the energy deposited by an ion leads to multiple cleavage of chemical bonds along the ion trajectory and formation of first generation of degradation products (molecular fragments, free radicals, ions and free electrons) exhibiting high chemical reactivity. Then the stable degradation products are formed by interaction of the reactive species with each other and with components of the polymer matrix as well. Typical degradation products are low mass molecular fragments and cross linked two- or three- dimensional structures. Relative fractions of these products depend on the polymer initial structure and composition and on the energy deposited by the ions [97-101]. Some of volatile degradation products (mostly light weight, hydrogen rich ones) may diffuse to the polymer surface and escape. This process leads to gradual carbonization of the polymer surface layer and creation of excessive free volumes [102-107]. The modified polymer surface may be attacked by aggressive species from ambient atmosphere (e.g. oxygen) and new structures may be formed on the polymer surface.

The implantation of low-to-medium energy ions changes only surface layer of polymers, with the thickness in nm-µm range, and preserves favorable bulk properties of polymers. By the ion implantation such surface properties as chemical structure [108-111], wettability [109,110,112], electrical conductivity [109,113-116], tribological properties [112,117,118] and bioactivity [18,111-113,119] can be changed in a manner which can be controlled by a proper choice of ion mass, energy and fluence.

Desorption of Volatile Degradation Products

Upon ion irradiation the polymer macromolecules are ionized and excited and a broad spectrum of degradation products of different mass and chemical activity are created. Low mass products, possessing higher mobility, may migrate to the sample surface and escape into evacuated implanter target chamber. The degassing rate can be determined by residual gas analysis. It was found that the main molecules and fragments liberated during ion irradiation of common polymers are H₂, CH₄, C₂H₂, CO, CO₂, CF, CF₂, CF₃. In “pure” hydrocarbons (PE, PP) hydrogen molecules may form about 90 % of released degradation products [102]. In polymers containing oxygen (e.g. PMMA, PI) beside hydrogen also carbon oxides are released in significant quantities (up to 50% of released products). Desorption of hydrogen molecules and hydrogen-rich fragments leads to gradual declining of hydrogen concentration in the polymer layer modified by the ion irradiation. Hydrogen depletion can be studied by any hydrogen sensitive depth profiling method (e.g. ERDA). In [120,121] the hydrogen and oxygen depth profiles were measured using standard ERDA and RBS techniques on different polymers (PE, PS, PI and PET) implanted with He, C, N, F, Ne, Ar and Xe ions at the energies from 20-200 keV to ion fluences varying from 10¹²-10¹⁷ cm⁻². The evolution of hydrogen depth profile with increasing ion fluence is illustrated in Figure 18. It was found that during initial stages of the ion implantation the hydrogen and oxygen contents in the irradiated surface layer decrease slowly but above some threshold fluence (typically 10¹³-10¹⁵ cm⁻²), which is a decreasing function of the ion stopping power, rather rapid decrease of H and O content is observed. At highest ion fluences the H and O contents achieves a saturated value which vary from 50-70 % of their original value in pristine polymer. Several
semiempirical models have been suggested describing hydrogen desoption during ion implantation (see e.g. [122]).

Figure 18. Depth profiles of hydrogen from polyimide (PI) implanted with 100 keV Kr ions to the fluences indicated. The horizontal line shows the hydrogen concentration in pristine PI. The profiles were determined from ERDA measurement performed with 2 MeV alpha particles [122]. The depth resolution was about 50 nm. It is seen that significant H depletion starts at the fluence of about $1 \times 10^{14}$ cm$^{-2}$.

Transport Processes in Ion-Irradiated Polymers

By ion irradiation a large number of highly reactive degradation products are produced during whole implantation process. Desorption of volatile degradation products leads to creation of excessive free volume which may facilitate penetration of gaseous species from residual atmosphere in the ion implanter. Reactive agents such as oxygen may react with degradation products and form new oxidized structures, which can be seen in RBS spectra of implanted samples. These structures may affect significantly several properties of the ion implanted polymers. The depth profiles of the incorporated oxygen were studied on polymers implanted with B, A, F, As, Sb and I ions [123-126]. In Figure 19 [125] the depth profiles of oxygen incorporated in PE during implantation of 150 keV Sb ions are shown as a function of the ion fluence. It is seen that oxygen penetrates through the implanted surface layer and is trapped within. With increasing ion fluence the oxygen content also increases up to some
critical fluence which varies from $10^{14}$ to $10^{15}$ cm$^{-2}$ for heavy and light ions respectively. At the critical fluence the oxygen content achieves a maximum. For still higher fluences the oxygen content declines probably due to destroying of the oxidized structures created in initial phases of the ion irradiation. At the maximum the total amount of trapped oxygen can be quite large. It was found that in polyethylene implanted with 150 keV F ions ($5 \times 10^{15}$ cm$^{-2}$) oxygen concentration in ion implanted layer achieves 25% of that of carbon.

Figure 19. Depth profiles of oxygen incorporated into the surface layer of PE implanted with 150 keV Sb ions to the following fluences: $2 \times 10^{13}$ (.), $1 \times 10^{14}$ (o), $2 \times 10^{14}$ (+), $1 \times 10^{15}$ (A) and $2 \times 10^{15}$ cm$^{-2}$ (♦) [125].

As can be seen in Figure 19 the oxygen depth profile is not homogenous and for low ion fluences it follows roughly the profile of ion electronic energy loss. For highest fluences the oxygen content decreases and its depth profile acquires bimodal form, with two concentration maxima, one near the sample surface and second, more pronounced, near the ion range. The bimodal depth profile gives evidence of deep structural changes induced by high fluence implantation.

**Surface Morphology of Ion-Implanted Polymers**

Ion-implanted polymers exhibit altered surface morphology, wettability, chemical affinity, bioactivity and electrical conductance. The observed changes are due to deep structural and compositional changes of polymer surface layer taking place during the ion irradiation. Surface morphology of pristine and modified polymers may be changed by different processes mentioned before, such as desorption of volatile degradation products through sample surface or preferential sputtering of amorphous and crystalline phases of polymers. The morphology can best be examined by AFM technique. Surface morphology of polyimide (PI) implanted with 90 keV N ions to the fluences from $1 \times 10^{14}$–$2 \times 10^{17}$ cm$^{-2}$ was
investigated by standard AFM [127]. It was found that the pristine PI exhibits rather diverse, bumpy surface with formations with typical dimensions of 200 nm and 2 nm in lateral and vertical directions respectively. The irradiation to $10^{14}$ cm$^{-2}$ leads to dramatic smoothing of the polymers surface but for increasing fluences the surface roughness increases again. The maximum roughness is observed at the fluence of $1 \times 10^{17}$ cm$^{-2}$ but for the fluence $2 \times 10^{17}$ cm$^{-2}$ the roughness decreases again probably due to progressive compaction of the polymer surface (see Figure 20).

![Figure 20. AFM deflection images of pristine polyimide (PI) and PI implanted with 90 keV N ions to fluences indicated [127].](image)

Occurrence of a graphitic phase on PI irradiated to the fluences $1 \times 10^{16}$ and $1 \times 10^{17}$ cm$^{-2}$ was proved by XRD measurement. Similar surface evolution was observed in [118] on PI implanted with 100 keV C and N ions to the fluences from $3 \times 10^{15}-1 \times 10^{17}$ cm$^{-2}$. In [128] PI was implanted with 40 and 80 keV Ar ions at high ion flux and to the fluences from $10^{14}-10^{17}$ cm$^{-2}$. At lowest fluence small craters at the ion impact sites were observed by AFM. With increasing ion fluence bubbles occur on the polymer surface and the surface roughness increases significantly. The bulb appearance is explained by effect of escaping Ar gas through the sample surface. Similar AFM examination was accomplished on PE implanted with 63 keV Ar ions and 155 keV Xe ions to the fluences from $1 \times 10^{13}-3 \times 10^{15}$ cm$^{-2}$ [117]. The irradiation to lowest fluence does not result in any significant change in the PE surface.
morphology but at higher fluences rather dramatic changes are observed. New structures appear on the PE surface and the surface roughness increases in comparison with that of pristine PE, the increase being larger for Ar ions. Great differences were observed between the topographic effects created by two different ions. The Ar ions produce rather fine but sharp surface structures while the structures produced by heavier Xe ions are broader and shallower. It appears that AFM technique is very useful for investigation of polymer surface morphology and its changes under ion irradiation. Specific phenomenon, i.e. ablation under ion irradiation was studied on PTFE, the polymer which is extremely radiation sensitive [129]. The PTFE samples were irradiated with 300 keV Ar ions to fluences from 1x10^{14}-1x10^{16} cm^{-2}. The thickness of ablated layer, determined by optical microscopy, was found to be an increasing function of the ion fluence; for the fluence of 1x10^{16} cm^{-2} about 14 µm of PTFE was removed by ion irradiation. XRD measurement shows gradual loss of PTFE crystalline phase with increasing ion fluence.

New Functional Groups Created by Ion Irradiation

Destruction of initial structure of polymers and the occurrence of new structures formed by ion irradiation can be followed using FTIR, UV-Vis and XPS spectroscopy. Structural changes of PTFE implanted with Ar ions to the fluences from 1x10^{14}-1x10^{16} cm^{-2} were examined by FTIR method [129]. It was found that new C=C and C=CF_2 bonds are produced together with C=O group, the formation of which indicate significant oxidation of the irradiated PTFE. The concentration of these new structures is an increasing function of the ion fluence. On the other hand, the irradiation to fluences above 3x10^{15} cm^{-2} results in a significant decrease of concentration of C-C bonds and CF_2 group. The decrease indicates splitting of C=C bonds and release of CF_2 fragments. At the same time the increase of absorbance in UV-Vis spectra with increasing ion fluence points to production and accumulation of conjugated double bonds in irradiated PTFE. In [118] gradual destruction of imide group (-CO-N-CO-) in PI irradiated with C and N ions to increasing ion fluences was observed from FTIR spectra. The destruction is accompanied by significant oxygen depletion in polymer surface layer which is observed in RBS spectra. Similar results were obtained in [130], where PET, polyetheretherketone (PEEK) and PI were irradiated with 150 keV Ar and 1.76 MeV He ions to the fluences from 10^{12}-10^{15} cm^{-2}. The compositional and structural changes were examined using RBS and ERDA techniques, UV-Vis spectroscopy and positron annihilation spectroscopy (PAS). Significant absorbance increase in UV-Vis spectra was observed on PEEK and PET irradiated with He ions and PET irradiated with Ar ions. The absorbance increases with increasing ion fluence. Also decline in hydrogen and oxygen concentration in the surface layer about 250 nm thick was observed on PET sample irradiated with Ar ions to the fluence of 1x10^{15} cm^{-2}. No such effect was initiated by He ions, probably due to their much lower stopping power.
7. Photochemical Modification of Polymers by UV Light-Irradiation

Similarly as the ion implantation or plasma irradiation described above, also the irradiation with UV light lea ded to splitting chemical bonds in the irradiated polymer and creation of radicals. The radicals then interacted mutually, and in the presence of oxygen and ammonia atmosphere, new chemical functional groups, such as C-H, C-O-, C=O and C-NH₂ as well as conjugated double bonds (C=C) and different free radicals were created. In PTFE, the newly formed groups often replaced F-atoms. These groups increased the surface polarity and wettability, which was positively correlated with the exposure time and well apparent on the originally highly hydrophobic PTFE surface. The UV light-irradiation of PET in acetylene atmosphere leadd to the formation of hydrogenated amorphous carbon (a-C:H) [7-11].

8. Cell Colonization on Materials Modified by Plasma Discharge, Ion Implantation or UV Light Irradiation

The changes in physicochemical properties of the material surface after irradiation with plasma, ions or UV light, such as formation of new chemical functional groups, increased surface polarity, wettability, electrical charge and conductivity as well as the formation of nano-sized irregularities on the material surface, are generally associated with the improved adhesion of various types of cells, including vascular endothelial and smooth muscle cells, osteoblasts, chondrocytes, fibroblasts or cells of the nervous system [7-11,18,40,132-134]. As the main underlying mechanism, improved adsorption of cell adhesion-mediating molecules to the material surface can be considered (Figure 4). On physicochemically modified surfaces, these molecules are adsorbed more homogeneously. In addition, their amount, spectrum and probably also the spatial conformation are appropriate for a good accessibility of active sites (i.e., specific amino acid sequences or saccharide-based ligands, see above) on these molecules by adhesion receptors on the cell membrane (Figures 5,6). The importance of spatial conformation of adsorbed molecules for the cell attachment is suggested on the Figure 6. It is apparent that the intensity of fluorescence, i.e. an indicator of the amount of collagen IV adsorbed on ion-implanted polyethylene samples, has an increasing tendency which is proportional to the increasing ion dose, while the number of initially adhered vascular smooth muscle cells reached its maximum at the medium ion doses and then strongly decreased. Thus, not only the absolute amount of the adsorbed proteins is important for cell adhesion, but also some other factors are needed - most probably the appropriate spatial conformation of the adsorbed molecules. In addition, it is known that on wettable surfaces, like those modified by ion-, plasma- or UV light-irradiation, the adsorbed cell adhesion-mediating molecules are flexible. The flexibility enables their active spatial reorganization, resorption and resynthesis by cells, which further contributes to the better accessibility of the active sites by the cell adhesion receptors [18,19,40,44-46]. On the other hand, a sufficient rigidity of the adhesion substrate is also required for a good cell adhesion and
Cell Colonization Control by Physical and Chemical Modification of Materials

spreading. If the material is too elastic, compliant, flexible and irreversibly deformable, it does not allow the anchorage of cells, even if the ligands for adhesion receptors are present in satisfactory amounts and accessibility, and are bound by these receptors. Such type of substrate cannot resist the cell tractional forces generated by the assembling cytoskeleton. For example, when collagen was adsorbed or covalently bound on glass or hard polyacrylamide gel (PAG), the vascular smooth muscle cells were normally spread with multiple streak-like focal adhesion sites and rich actin cytoskeleton, and were viable. However, if soft PAG or pure collagen gel was used, the cells remained round (without signs of spreading and assembly of adhesion apparatus), and underwent apoptosis [40,54].

Not only the cell number, but also the cell spreading area was generally enlarged on ion-, plasma- or UV light-irradiated polymers (Figure 21). The cells adhered by more numerous and larger focal adhesion plaques and contained a well developed actin cytoskeleton. As revealed by ELISA, also the concentrations of focal adhesion proteins vinculin, talin, paxilin and alpha-actinin were usually increased in comparison with cells growing on non-modified polymers (Figure 8).

Figure 21. Morphology of rat aortic smooth muscle cells on day 1 (A, B) and 3 (C, D) after seeding on pristine unmodified PE foils (A, C) or foils irradiated with \(3 \times 10^{13}\) ions C\(^+\) cm\(^{-2}\) (B) or \(3 \times 10^{14}\) ions O\(^-\) cm\(^{-2}\) (D); energy 150 keV. Stained with hematoxylin and eosin, microscope Opton Axioplan, Leica, Germany. Bar=100 µm.

The cells were more resistant to the proteolytic enzyme-mediated as well as spontaneous detachment. They were able to persist on the modified polymer surfaces in confluent, viable and metabolically active state for several months [7-11,18,19,40,132-134].

Also the proliferation activity of cells was often increased on the ion-, plasma- or UV light-modified surfaces. However, in some cases, when the initial adhesion and spreading of cell was very high, the proliferation activity was similar or even lower than in cell on non-modified
surfaces. It is well known that the cell behavior, such as migration, proliferation, differentiation, appropriate functioning or long-term viability, is regulated by the extent of initial cell adhesion. The size and shape of cell spreading area, as well as the number, size, shape and distribution of focal adhesion plaques are decisive for further migratory, proliferative and differentiation behavior of anchorage-dependent cells (Table 2). If this extent is very small (i.e. attachment of round cells without formation of focal contacts and spreading), the cells usually do not survive. At the intermediate adhesion strength, the cells are most active in migration and proliferation, and if the cell-material contact area is very large with multiple well-developed focal adhesion sites associated with a rich cytoskeleton, the cells tend to skip the proliferation phase and enter sooner the differentiation program (Table 2, Figure 8). The latter cell behavior is advantageous for construction of bioartificial vascular prostheses, where a soon maturity of both endothelial and smooth muscle cells is required in order to prevent thrombogenicity and immunogenicity of the cell-material constructs and excessive proliferation of vascular smooth muscle cells, often leading to restenosis and even full obliteration of vascular prostheses [18,19,31,40,41,134].

9. Patterned Surfaces and Grafting Bioactive Molecules

Physicochemical modifications of the polymer surfaces by ion-, plasma- or UV light-irradiation can be advantageously applied for the construction of micropatterned surfaces used for regionally selective adhesion and growth of cells. Directed cell adhesion and growth is needed for some specific tissue engineering applications, as well as construction of microarrays used in advanced geomics and proteomics. Typically, the polymers are irradiated through metallic mask with openings of various sizes and shapes. In addition, if the openings are relatively small, allowing the adhesion and spreading of one individual cell, the size and shape of these openings can control the size and shape of cell adhesion area, and thus the following cell behavior. In our studies, the most selective cell adhesion and growth of cells on modified spots was obtained using UV light irradiation in ammonia atmosphere (Figure 22) [10,11].
Cell Colonization Control by Physical and Chemical Modification of Materials

The presence of free radicals, double bonds and various chemical functional groups on ion-, plasma- or UV light-modified surfaces may be also utilized for chemical bonding of other substances, particularly various bioactive molecules, such as amino acids, oligopeptidic ligands for cell adhesion receptors or active sites of various enzymes and hormones. When polymers modified by UV light in ammonia atmosphere or by Ar plasma were grafted with amino acids, such as glycine, alanine or leucine, the cell adhesion and growth were further enhanced (Figure 23) [8,9].
Although the single amino acids cannot bind the adhesion receptors on cells, they provide other chemical functional groups which may improve the adsorption of cell adhesion-mediating molecules on the material surface. Similar effect was observed on ion-implanted polyethylene grafted with the Arg-Gly-Asp (RGD) sequence [135]. Although this tripeptide represents a minimum ligand for integrin adhesion receptors, it was not probably bound directly to the receptors, because it was attached to the material surface relatively tightly and rigidly. In order to facilitate binding to adhesion receptors, the oligopeptides must be tethered on the material surface through a flexible chain ensuring good accessibility of these ligands to cell adhesion receptors, together with an appropriate spatial conformation of these ligands (i.e., circular of loop-like shape). A specific binding of a RGD-containing oligopeptide, i.e. GRGDSG, was achieved on polylactide-based surfaces, where the oligopeptides were
tethered through polyethylene oxide (PEO) chains of an appropriate length and concentration (Figure 24) [41].

Figure 24. Scheme of a synthetic biodegradable polymer for tissue engineering, endowed with ligands for cell adhesion receptors. PLLA: poly-L-lactide, representing the bulk material of a newly constructed bioartificial tissue, e.g. vascular wall. PDLLA: poly-DL-lactide chains for copolymerization with polyethylene oxide (PEO) in order to prepare a bioinert surface. Due to its extreme hydrophilia, PEO do not allow spontaneous protein adsorption and aberrant cell adhesion. In addition, flexible PEO chains ensure the accessibility of ligands for cell adhesion receptors. These ligands could be represented by various amino acid sequences, such as RGD-containing oligopeptides, as well as their cooperating sequences like PHSRN [41].

These chains not only ensured a good accessibility of GRGDSG by integrin receptors but provided the material surface with a bioinert background preventing aberrant protein adsorption and cell adhesion, which is due to the extreme hydrophilia of PEO. As a result, the cell adhesion and growth could be fully controlled by the type, concentration and spatial distribution of oligopeptidic and other adhesion ligands on the material surface [38-41]. The effects of adhesion oligopeptides tethered on the material surface can be enhanced in serum-free medium, because the absence of serum minimizes the potential adsorption of cell adhesion mediating ECM molecules on the regions with oligopeptides. Vascular smooth muscle cells cultured on the GRGDSG-grafted surfaces in a chemically-defined serum-free medium in our experiments were able to synthesize DNA and proliferate (Figure 25) [41].
Figure 25. A-D: Immunofluorescence staining of vinculin in vascular smooth muscle cells on day 3 after seeding on polymeric surfaces (medium supplemented with 10% fetal bovine serum). A: poly(DL-lactic acid), PDLLA; B: block copolymer of poly(DL-lactic acid) and poly (ethylene oxide) (PEO), PDLLA-b-PEO; C, E: PDLLA-b-PEO with 5% GRGDSG-PEO-b-PDLLA; D, F: PDLLA-b-PEO with 20% GRGDSG-PEO-b-PDLLA. E, F: Immunoperoxidase staining of bromodeoxyuridine (arrows) incorporated into DNA newly synthesized in vascular smooth muscle cells cultured for 3 days in serum-free medium on PDLLA-b-PEO with 5% (E) or 20% (F) GRGDSG-PEO-b-PDLLA. Cells counterstained with light green. Bar=100 µm [41].

10. Modification of Materials by Deposition of Organic or Inorganic Layers
The bioactivity of various materials designed for implantation into the human body could be also enhanced by coating with various soft or hard, degradable or durable layers.

The soft degradable layers are represented by various extracellular matrix molecules and proteins involved in tissue regeneration, such as collagen, laminin, fibronectin, fibrin and their combinations [31,48,49]. Controlled assemblies of these molecules are suitable e.g. for modification of the inner surface of vascular prostheses in order to improve their endothelialization (Figure 26).

Figure 26. Reconstruction of the tunica intima on the inner surface of a clinically used polyethylene terephthalate vascular prosthesis. A: non-modified inner surface of the prosthesis, B: immobilization of defined assemblies of protein molecules (e.g., collagen+laminin or collagen+fibrin) on the inner surface of the graft, C: immunofluorescence of von Willebrand factor, a marker of the identity and differentiation of vascular endothelial cells, in human saphenous vein endothelial cells in cultures on the inner surface of a prosthesis coated with collagen and laminin, D: detail of a layer of endothelial cells growing on a layer of collagen and fibrin. Note well developed talin-containing focal adhesion plaques. A, B: conventional optical microscope, C, D: confocal microscope Leica DM 2500 [30,31].

These molecules represent a natural source of ligands for cell adhesion receptors in an appropriate amount, spectrum, spatial conformation as well as the presence of cooperating sequences. On the other hand, allogeneous or xenogenous ECM molecules can be immunogenic or associated with a risk of pathogen transfer. These problems could be avoided by isolation of autologous fibrin from the patient’s blood in a sufficient quantity. Thin protein layers could be also used for coating of the inner surfaces of three-dimensional scaffolds. In other words, the walls of the pores inside the material could be coated with a
thin layer of cell adhesion-mediating molecules without filling the entire volume of pores, which could improve the ingrowth of cells (Figure 27).

Figure 27. Human osteoblast-like MG 63 cells in cultures on porous (A) or fibrous (B) poly(L-lactide-co-glycolide) scaffolds. A: A summarizing picture of horizontal optical sections. The depth of cell ingrowth into the pores (average pore diameter of 400-600 mm) is indicated by spectral colors (blue: 0–60 mm, green: 80–160 mm, yellow: 180–220 mm, orange: 240–300 mm, red: 320–400 mm, violet: 420–480 mm). Day 14 after seeding, cells stained with propidium iodide. B: cells grown for 4 days in static culture followed by 2 days in dynamic perfusion cell culture system. Cell membrane stained with Texas Red C2-maleimide and the nuclei counterstained with Hoechst #33342. Leica TCS SP2 confocal microscope, objective 5x (A) or 10x (B) [37].

The hard layers are represented with a wide range of coating based on carbon, metals, ceramics, various oxides (TiO$_2$, SiO$_2$) and nitrides (ZrN) and their combinations [14,16,24,47,136-143]. These layers can strengthen the material surface, especially in materials designed for hard tissue surgery, such as construction of bone and joint replacements and bone fixation. In addition, these layers can smooth various irregularities on the material surface, which can hamper the adhesion, spreading and growth of cells [14-16,136,138,140,143]. For a more pronounced surface smoothing, the deposition of layers can be combined with grinding and polishing of the material surface (Figure 28). In addition to the surface smoothing, the beneficial effects of the deposited layers on the cell adhesion are often mediated by the presence of oxygen-containing groups and appropriate wettability, and especially by the nanostructure of some of these layers, i.e. the presence of particles less than 100 nm. The nanostructured surfaces resemble, at least to a certain degree, the architecture of physiological adhesion substrates, such as extracellular matrix, which is composed from nanoscale proteins, and in the case of bone, also hydroxyapatite and other inorganic nanocrystals [16,17,24-27]. From this point of view, carbon nanoparticles, such as fullerenes, nanotubes and nanodiamonds, may serve as important novel building blocks for creating artificial bioinspired nanostructured surfaces for bone tissue engineering.
Fullerenes $C_{60}$ were found to promote proliferation and differentiation of chondrocytes and to enhance the production of specific large proteoglycan ECM molecules, typical for cartilage, in rat embryonic limb bud cells in culture [144]. In a line of human epidermoid carcinoma cells, fullerenes protected cells from anoikis, i.e., apoptosis due to adhesion deprivation, by a mechanism supporting the formation of focal adhesion plaques, assembly of the actin cytoskeleton and cell spreading [65]. Enhanced attachment and spreading of platelets was also found on a polyurethane surface grafted with fullerene $C_{60}$ molecules [145]. Fullerene $C_{60}$ layers formed on polystyrene culture dishes by the evaporation of methanol from colloidal $C_{60}$-methanol suspensions, promoted adhesion, proliferation and assembly of an actin cytoskeleton in several lines of normal and malignant breast epithelial cells [146]. Similarly, non-cytotoxicity of nanodiamonds (2 to 10 nm in size) and their supportive effect on cell adhesion and growth has been demonstrated on a variety cell types, including neuroblastoma cells, macrophages, and keratinocytes [147].

Recently, a very interesting finding has been reported that nanostructured surfaces can promote preferential adhesion and growth of osteoblasts over other “competitive” cell types, mainly fibroblasts (which can cause fibrous encapsulation and loosening of bone implants). It is considered that the underlying mechanism is a higher adsorption of vitronectin, an ECM
protein preferred by osteoblasts, to the nanostructured surfaces [24-27]. In future, carbon and other nanoparticles could be integrated into so-called “three-dimensional” matrices for constructing bone replacements, i.e. materials with a porous or scaffold-like architecture hierarchically organized at both micro- and nano-size level according principles observed in natural tissues and organs. For example, carbon nanoparticles could decorate the walls of micropores in the material and thus create a nanopattern promoting the ingrowth of osteogenic cells inside the scaffolds (Figure 27) [17,148]. In addition, carbon nanoparticles, especially, nanotubes can reinforce synthetic polymers designed for bone tissue engineering. Nanotubes can resemble nanofibres of collagen and other ECM proteins of the bone, and together with other carbon nanoparticles, also hydroxyapatite and other inorganic crystals in the bone [25,26]. Furthermore, carbon nanotubes can be used effectively for electrical stimulation of osteoblasts, which has been reported to promote their proliferation, differentiation, production of mineralized bone matrix and thus healing of the damaged bone [25].

Figure 29. Human osteoblast-like MG 63 cells in cultures on material surfaces modified with carbon nanoparticles. A: fullerene \(C_{60}\) layers deposited on carbon fibre-reinforced carbon composites (CFRC), B: fullerene \(C_{60}\) layers deposited on microscopic glass coverslips, C: terpolymer of polytetrafluoroethylene, polyvinylidifluoride and polypropylene, mixed with 4% of single-wall carbon nanohorns, D: the same terpolymer with high crystalline electric arc multi-wall nanotubes, E: diamond layer with hierarchically organized micro- and nanostructure deposited on a Si substrate, F: nanocrystalline diamond layer on a Si substrate. Standard control cell culture substrates were represented by a PS culture dish (G) and microscopic glass coverslip (H). Immunofluorescence staining on day 2 (A) or 3 (B-H) after seeding, Olympus epifluorescence microscope IX 50, digital camera DP 70, obj. 20x, bar: 100 \(\mu m\) (A, C, D, G, H) or 200 \(\mu m\) (B, E, F) [16].
Promising results on carbon nanoparticles were obtained also in our earlier experiments. Fullerenes C_{60}, deposited on carbon fibre-reinforced carbon composites or microscopic glass coverslips, as well as nanocrystalline diamond on silicon substrates, promoted adhesion, growth, viability, mitochondrial activity and differentiation of human osteoblast-like MG 63 cells, usually in an extent similar as on standard cell culture substrates represented by polystyrene cell culture dishes or microscopic glass coverslips. On a terpolymer of polytetrafluoroethylene, polyvinylidifluoride and polypropylene, i.e. a material promising for construction of bone implants, which was mixed with single- or multiwalled carbon nanotubes, the colonization with MG 63 cells was even significantly better than on the unmodified terpolymer (Figure 29). At the same time, the presence of nanotubes did not increase the immune activation of cells, measured by the concentration of ICAM-1 per mg of protein [16].

The interactions of fullerenes, carbon nanotubes and nanodiamonds with biological systems can be further enhanced by functionalization of these molecules with various atoms, chemical groups or molecules (e.g., hydrogen, halogens, aldehydic, ketonic, alcoholic, carboxylic acid/ester moieties, amine groups, amino acids), which usually renders them soluble in water, and could stimulate the adsorption of cell adhesion-mediating ECM molecules [148-151]. The carbon nanoparticles could be also functionalized by ligands for cell adhesion receptors, such as RGD-containing oligopeptides or KRSR preferred by bone cells.

An interesting property of fullerenes and nanotubes, especially after derivatization, is the tendency to form self-assembling surface and bulk morphologies, such as patterned fullerene lipid films containing C_{60} [152], self-assembling chemisorbed amino octane thiol based-monolayers functionalized with fullerene C_{60} [153], self-assembling nanotube structures [151] or large scale stripe and circular patterns in transition metal/C_{60} composites [154]. These self-assembling layers are often organized in micro- and nano-dimensional patterns, and might serve as templates for spatially controlled adhesion and directed growth of cells. The microstructured surfaces can be also created by the deposition of carbon nanoparticles, e.g. fullerenes, through a metallic mask (Figure 22 D).

11. Conclusion

The bioactivity of artificial materials designed for biomedical use can be enhanced by various physico-chemical modifications, such as irradiation by plasma, ions or UV light in reactive atmospheres. These modifications lead to the formation of new structures, such as chemical functional groups, radicals and double bonds, which improve spontaneous adsorption of cell adhesion-mediating molecules from the culture media or body fluids. In addition, these structures can be utilized for functionalization of the materials with various biomolecules, e.g. oligopeptidic ligands for cell adhesion receptors. Another important tool for construction of bioactive materials is their coating with various soft or hard layers, especially defined assemblies of cell adhesion-mediating molecules or inorganic nanostructured films composed e.g. from ceramic, metallic or carbon nanoparticles. Thus,
physical and chemical modifications of the material surface are of a great perspective for the future development of the biomaterial science and tissue engineering.

Acknowledgements

The work was supported by the Ministry of Education of the Czech Republic (Research program No. LC 06041), Academy of Sciences of the CR (Research programs No. KAN400480701, KAN101120701, A 5011301 and 1QS500110564) and the Grant Agency of the CR (Grants No. 204/06/0225 and 101/06/0226). The authors thank Mrs. O. Kesselová and Mrs. I. Zajanová for their technical assistance.

References

Cell Colonization Control by Physical and Chemical Modification of Materials


Cell Colonization Control by Physical and Chemical Modification of Materials