Investigations towards 2D and 3D cell culture devices based on nanocrystalline diamond layers

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Abstract

This thesis aims to study advanced biological applications of nanocrystalline diamond layers with a special focus on the interfacial situation between biomaterials and biological systems. Specifically, nanoscopic interfacial water layers have been investigated in laboratory experiments on nanocrystalline diamond layers and other model substrates by their modulation with low-level laser light. Based on the results, two novel kinds of cell culture devices coated with nanocrystalline diamond have been developed. First, the 3D diamond Petri dish (strawberry patterned diamond), which has been applied to study the assembly of stem cells in embryoid bodies. Second, a new generation of 2D cell culture devices with improved biocompatibility based on nanocrystalline diamond. Furthermore, new methods in nanomedicine, including a novel anti-cancer therapy model, inspired by the results of the experiments exploring nanoscopic interfacial water layers are presented.
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1 Introduction

Biomaterials science addresses the application of non-viable and/or viable materials in the biomedical devices intended to interact with the biological systems. It is a multidisciplinary research field encompassing aspects of physics, chemistry, biology, medicine, materials science and engineering. In principle, the study of a biomaterial consists of three basic building blocks: the solid material substrate, the interface between the material and biological system as well as the biological component. Since a material interacts with its immediate surrounding environment through the interface, one key factor that determines the success or failure of a biomaterial’s application is the material-biosystem interface, which is a complex and challenging issue with respect to sophisticated interactions between the biomaterial and the biological environment.\textsuperscript{1-4}

Diamond is an allotropic form of carbon, which is one of the most basic elements of living organisms. It is a fascinating biomaterial and attracts more and more attention for scientific research and industrial applications. Regarding the physical and mechanical properties, diamond is a robust material with impressive performance. In view of the chemical properties, diamond is extremely inert. Furthermore, diamond is resistant to the corrosive biological environment. Since nanomaterials – both particles and the related surfaces – present similar scales to biological molecules and systems yet can be engineered to have various functions, they are potentially useful for biomedical applications.\textsuperscript{5} There are two main manifestations of diamond-based nanomaterials. The first are nanocrystalline diamond layers, which are often used as biosensors,\textsuperscript{6-8} and as coatings on medical devices including biocompatible implants such as orthopedic implants,\textsuperscript{9,10} artificial heart valves,\textsuperscript{11} and medical instruments such as surgical blades.\textsuperscript{12,13} The purpose of the coatings is to extend the lifetime, or to chemically seal the surfaces. The second are diamond nanoparticles, which are widely used as drug carriers, for example in combination with chemotherapeutant to destroy
cancer cells\textsuperscript{14-17}, as well as nondestructive biomarkers for imaging cells and tissues\textsuperscript{18-20}. The aim of the thesis is to study biological applications of nanocrystalline diamond layers with a special focus on model biointerfaces, and subsequent transfer of the spin-off result into the intracellular space.

First part of the thesis deals with the interface issues with the emphasis on the structural properties of nanoscopic interfacial water layers due to their important roles in the bio-related systems that are rich in surfaces and interfaces. Nanoscopic interfacial water layers prevail on all the material surfaces both in air and under water. They have a molecular structure quite different from that of bulk water. Because of their potential implications in the biological activities, interfacial water layers have received much attention from researchers from various disciplines. In 1971, Szent Györgyi presented the idea of the fundamental position of interfacial water layers in many biological processes and evolution\textsuperscript{21}. Currently, interfacial water layers have been regarded as the informational blueprints of the underlying surfaces\textsuperscript{22}. Water-water interaction has been recognized to be fundamental in the process of biological responses to the artificial materials\textsuperscript{22,23}. Importantly, the smaller the object is, the more important roles the interfacial water layers play. A master-slave concept has been introduced. Interfacial water layers slave the encapsulated proteins, therefore they control the proteins and cells function and state\textsuperscript{24}.

The potential of nanoscopic interfacial water layers in biosystems and in biological activities is enormous, however, many of their implications have not yet been verified, due to the lack of suitable experiments which could provide insights into their intrinsic structural properties. Considering the properties of the extreme chemical and biological inertness as well as the controlled surface roughness, nanocrystalline diamond provides a unique platform to investigate nanoscopic interfacial water layers, which are most subtle and highly sensitive to observation. In this work, new methods are employed to probe and characterize nanoscopic interfacial water layers on nanocrystalline diamond as well as other hydrophobic and hydrophilic model surfaces.
The second part of the thesis provides new insight into the biological applications of nanocrystalline diamond layers; they are based on the study of the interface addressed in the first part of the thesis. Nanotechnology offers the possibility of coating varieties of substrates, including the complicated surfaces with homogeneous layers of nanocrystalline diamond. In biomedical applications, diamond coatings can be applied not only on medical devices but also on cell culture devices routinely used in life science laboratories. The common thread in these diverse applications is the unique character in the interaction between the diamond surfaces and biological systems.

The cell’s behavior, such as proliferation, apoptosis, adhesion and migration is controlled through numerous processes of the intracellular signaling events that are normally triggered by the receptors on the cell surface. In the biological applications of nanocrystalline diamond layers, once the diamond is placed into the biological environment, the cell-material recognition process starts. Notably, on the one hand, the first contact between the cells and the material is their contact to the nanoscopic interfacial water layers sealing their surfaces. On the other hand, diamond, more precisely, hydrogen-terminated nanocrystalline diamond is masked by nanoscopic interfacial water layers with very high organization and stability – one result from the first part of the thesis – which would act as damping elements softening the cell-material contact and regulate the cells’ response from the first contact events. From this perspective, this work firstly develops an innovative 3D culturing method for stem cell research, specifically, by applying the principles of biomimetic thinking, and secondly leads to a new generation of 2D cell culture devices with improved cell performance. Both applications are based on hydrogen-terminated nanocrystalline diamond layers.

The third part of the thesis concentrates on the nanoscopic interfacial water layers in the intracellular space. Cells are crowded with organelles and macromolecules, resulting in numerous surfaces and interfaces inside of the cells.\(^{25,26}\) It has been reported that the ratio of interfacial water to total water in the cell is about 30%; and
the ratio is much higher in certain organelles, for instance, mitochondria. In addition, as described in the first part of the thesis, a technique of modulating the molecular structure of the nanoscopic interfacial water layers by using low-level laser light has been developed. On this foundation, this work establishes a new approach to transport small molecules, such as drugs or nutrients, across the cell membrane by using transmembrane convection induced by the structural changes of the intracellular nanoscopic interfacial water layers in response to the low-level laser light irradiation – the principle of the advanced methods in nanomedicine.

In summary, in this thesis I focus on the study of advanced biological applications of nanocrystalline diamond layers, which emerge from the exceptional properties of the interfacial mediator prevailing on the diamond surfaces: the nanoscopic interfacial water layers. New methods in nanomedicine are inspired by the transfer of a spin-off result obtained from exploring the mediator in the laboratory experiments performed on the diamond model surfaces into the intracellular space. This is a multidisciplinary work based on engineering science principles and advanced tools in nanotechnology. It lays emphasis on materials science and subsequent applications in biomedicine in general and nanomedicine in particular.

The thesis is organized as follows. Chapter 2 outlines the solid materials: nanocrystalline diamond layers. Their growth method and characterization used in this thesis are described. Chapter 3 investigates the interface issues, which connect the materials and biological systems. Nanoscopic interfacial water layers are discussed in details. Four different experimental methods are used to explore their properties. Chapter 4 starts with the standard biological characterization of different nanocrystalline diamond layers and goes on to discuss the roles of interfacial water layers during the first contact events. Two biological applications of nanocrystalline diamond layers are then developed: one is to culture 3D embryoid bodies for stem cells research; the other is to improve cell performance especially for sensitive and precious cells, such as stem cells, primary cells and embryos. Chapter 5 presents the
new methods in nanomedicine, which is inspired by the study of the biological applications of nanocrystalline diamond layers. A novel anti-cancer therapy is described based on the modulation of the intracellular nanoscopic interfacial water layers by low-level laser light.

The cell culture experiments have been performed within the cooperative research project Bionic (Landesstiftung Baden-Württemberg) at the Institute for Biological Interfaces in Karlsruhe Institute of Technology (KIT). I am very grateful to Dr. Tim Scharnweber and Dr. Alexander Welle for this opportunity.
2 Materials: nanocrystalline diamond layers

Diamond is an ancient gemstone material with a variety of outstanding properties, exhibiting the highest hardness (~100 GPa), highest Young’s modulus (~1200 GPa), highest thermal conductivity (20 W cm\(^{-1}\) K\(^{-1}\)) of all materials known, as well as absolute chemical inertness and biocompatibility.\(^{28-30}\) The unique combination of these remarkable properties makes diamond a particularly interesting material for scientific research and industrial applications.

However, the high costs of natural diamonds restrict the study and applications of diamonds. Therefore, many research efforts have been made for synthesis of artificial diamonds. Nanotechnology offers the possibility of synthesizing diamonds in the form of nanocrystalline diamond layers with comparable or better properties than natural diamonds, for instance, the designable structure, which opens new horizons for applications.

Section 2.1 gives a brief introduction of the atomic structure, properties and the related applications of diamond. Section 2.2 and 2.3 present the synthesis and characterization of nanocrystalline diamond layers as well as three variations with hydrogen, oxygen and fluorine surface termination.

2.1 Introduction to diamond

The carbon atom has an electron configuration of 1s\(^2\)2s\(^2\)2p\(^2\). Depending on different hybridization of s and p valence orbital, there are different carbon allotropic forms, such as diamond, graphite, amorphous carbon, fullerenes and carbon nanotubes. The physical properties of these allotropic forms vary widely.

The diamond crystalline unit cell can be regarded as a variation of a face centered cubic lattice structure with additional carbon atoms occupying half of the tetrahedral
holes, as shown in Figure 2.1.1. Each carbon atom is surrounded by four adjacent atoms, forming a bond angle of 109.5° and a bond length of 0.154 nm.

![Diamond crystalline lattice](image)

**Figure 2.1.1** Diamond crystalline lattice.

In diamond, sp³ orbital hybridization is formed, resulting in strong covalent bonding between carbon atoms (347 kJ mol⁻¹). This leads to many extraordinary properties of diamond. Table 2.1.1 shows a list of some properties of diamond under ambient conditions.

<table>
<thead>
<tr>
<th>Property</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hardness</td>
<td>~ 100 GPa</td>
</tr>
<tr>
<td>Young’s modulus</td>
<td>~ 1200 GPa</td>
</tr>
<tr>
<td>Thermal conductivity</td>
<td>20 W cm⁻¹ K⁻¹</td>
</tr>
<tr>
<td>Resistivity</td>
<td>~ 10¹⁶ Ω cm</td>
</tr>
<tr>
<td>Band gap</td>
<td>5.47 eV</td>
</tr>
<tr>
<td>Electrons mobility</td>
<td>2400 cm² V⁻¹ s⁻¹</td>
</tr>
<tr>
<td>Holes mobility</td>
<td>2100 cm² V⁻¹ s⁻¹</td>
</tr>
<tr>
<td>Electrical breakdown field</td>
<td>~ 2 × 10⁷ V cm⁻¹</td>
</tr>
<tr>
<td>Optical transparency</td>
<td>From UV to IR</td>
</tr>
<tr>
<td>Low friction coefficient</td>
<td></td>
</tr>
<tr>
<td>Very resistant to chemical corrosion</td>
<td></td>
</tr>
<tr>
<td>Good biocompatibility</td>
<td></td>
</tr>
</tbody>
</table>

**Table 2.1.1** Properties of diamond under ambient conditions (room temperature and atmospheric pressure).
The properties of the highest hardness, highest Young’s modulus and highest thermal conductivity (see table 2.1.1) make diamond an interesting material for mechanical applications, such as cutting and grinding tools. Whereas diamond is a good insulator due to the wide band gap (5.47 eV), it is possible to dope diamond to be n-type (for instance, using boring) or p-type (for instance, using phosphorous) semiconductor, which makes diamond a suitable candidate for electronics devices. In addition, the extreme chemical inertness and good biocompatibility brings diamond into the focus of the biological fields. Details are described in Chapter 4.

2.2 Nanocrystalline diamond layers: growth and characterization

In nature, most diamonds are formed during redox reactions under high-pressure high-temperature conditions at the depths from 140 to 200 km in the Earth’s mantle, or deeper (superdeep diamonds) and brought to the Earth’s surface by the complex volcanic magmas. In the early stages of man-made diamond research, scientists tried to synthesize diamond from other allotropic forms of carbon, for instance, graphite, by simulating the environment for natural diamonds formation. The first reproducible synthetic diamonds are made with high-pressure high-temperature (HPHT) technique and reported in 1955. The yielding diamonds are with the size ranging from micrometer to millimeters in the form of single crystals. Almost at the same time, there is another major breakthrough in the synthetic diamonds research, that is, diamonds grow under relatively low pressure from a mixture of hydrocarbon gas via the chemical vapor deposition (CVD) process. The international explosion of CVD diamond research starts in early 1980s. Several publications describe the growth of diamond layers on silicon substrates at temperatures of ~ 900 °C and pressures of ~ 5 kPa from a mixture gas of methane and hydrogen using hot filament or microwave plasma enhanced CVD technique. Since then, the CVD diamond technique spread
worldwide and has been intensively studied by increasing numbers of research groups. The CVD method provides the synthetic diamonds with relatively simple apparatus and affordable costs. Importantly, by controlling the growth conditions, this technique allows the growth of diamond layers over large areas and on varieties of substrates with different surface and bulk properties. As a consequence, the CVD produced diamond layers ranging from polycrystalline to nanocrystalline are in the focus of extensive research and promise wide-ranging applications in different dimensions.

2.2.1 The chemical vapor deposition (CVD) technique

Figure 2.2.1 is a phase diagram of carbon showing the temperature and pressure conditions for diamond growth. As can be seen, CVD diamond can be synthesized under relatively low temperature and pressure compared to HPHT diamond. The fundamental principle of the CVD diamond technique is to use gas-phase carbon species to deposit diamond lattice structure on the substrate. A key for the success of CVD diamond synthesis is to simultaneously suppress the formation of sp$^2$ graphite. Normally, in the CVD chamber, methane is supplied as carbon source and high concentration hydrogen is to control the quality of diamond layers, as atomic hydrogen is found to etch sp$^2$ much faster than sp$^3$ carbon, resulting that diamond is the predominant carbon phase.
Figure 2.2.1 Phase diagram of diamond and graphite.\textsuperscript{13}

The formation of CVD diamond layers involves the steps for a typical crystal growth process, including nucleation and propagation. The nucleation process is to initiate the sp\textsuperscript{3} diamond lattice. The growth of CVD diamond layers on natural diamond substrates is the extension of the diamond lattice during the deposition process, which is called homoepitaxial growth. In the case of heteroepitaxial growth, in other words, when a non-diamond substrate is used, the initial diamond seeding on the substrate is essential to create a template for the diamond growth. This can be achieved by abrading the powder of diamond nanoparticles on the substrate manually or by immersing the substrate into a solution containing diamond nanoparticles with ultrasonic agitation. The ultrasonic method allows the deposition of diamond layers on the substrates with complicated shapes.

In the process of CVD diamond growth, the activation and dissociation of the gas phase reaction requires a heat source, such as a hot filament or a microwave plasma reactor. In a typical hot filament reactor, a coil of metal, for instance, tungsten is heated to the temperature of 1800 – 2200 °C. A substrate close to the filament is at the temperature of 500 – 1000 °C. The ratio of CH\textsubscript{4} to H\textsubscript{2} is around 1% with the total
pressure of 2 – 20 mbar.\textsuperscript{39} Figure 2.2.2 is a schematic diagram of a hot filament CVD diamond process and a photo of the CVD chamber in our institute.

![Schematic diagram of a hot filament CVD diamond process and a photo of the CVD chamber.](image)

**Figure 2.2.2** Schematic diagram of a hot filament CVD diamond process (top) and a photo of the CVD chamber (bottom). (based on Ref\textsuperscript{40})

So far, the mechanism of the CVD diamond growth has not been fully understood.\textsuperscript{41} The current knowledge is that atomic hydrogen and other reactive hydrocarbon radicals are dissociated in the gas phase and after a series of surface reactions the gas phase carbon is incorporated into the bulk diamond structure in the present of hydrogen rich environment.\textsuperscript{29}
The properties of the CVD diamond layers, such as the surface morphology and mechanical performance vary a lot depending on the different initial diamond nucleation density and the CVD growth conditions, including the input gas mixture, filament temperature, substrate temperature, and pressure. Nanocrystalline diamond layers with grain sizes from a few nanometers to hundred nanometers can be achieved by the adjustment of the above-mentioned parameters.

The CVD nanocrystalline diamond coatings has been successfully applied as cutting tools with outstanding and long-lasting sharpness, such as diamond blades, as well as micromechanical parts with wear-resistant properties, for instance, diamond gear wheel for watch industry. The biological applications of nanocrystalline diamond layers will be in details discussed in chapter 4.

### 2.2.2 Characterization of nanocrystalline diamond layers

In this thesis, nanocrystalline diamond layers deposited on silicon (100) wafers are employed for the study of nanoscopic interfacial water layers as well as biological applications. The layers were fabricated from a gas mixture of H₂, CH₄, N₂ and O₂ in a hot filament CVD reactor equipped with tungsten filament. Depending on the initial seeding method (manually or ultrasonically excited) and the CVD chamber growth conditions, different nanocrystalline diamond layers were achieved reproducibly with the thickness of 1 – 6 µm, average grain size of 15 – 250 nm and root mean square roughness of 18 – 37 nm. The relevant parameters will be communicated in each individual experiment.

Figure 2.2.3 represents scanning electron microscopy (SEM) images revealing the morphology of different nanocrystalline diamond layers with the average grain size of 250 nm (left) and 15 nm (right).
Figure 2.2.3 SEM micrographs of different nanocrystalline diamond layers.

2.3 Different surface terminations of nanocrystalline diamond layers

The hot filament CVD diamond layers are grown in the hydrogen-rich environment and the diamond lattice is terminated with hydrogen. In addition, nanocrystalline diamond layers can be stably terminated with certain elements on the surfaces, such as hydrogen, oxygen and fluorine. In this study, the surface terminations were achieved by short exposure (15 min) to hydrogen, oxygen and fluorine plasma, respectively. The chemical termination does not alter the morphology of the layers, but the electron affinity properties. Contact angle measurements reveal that the non-terminated and hydrogen-terminated nanocrystalline diamond surface is hydrophobic, whereas the oxygen and fluorine-terminated surface is very hydrophilic and very hydrophobic. For instance, for the non-terminated and different terminated (hydrogen, oxygen and fluorine) diamond samples with identical surface morphology (average grain size of 250 nm and root mean square roughness of 37 nm), the contact angle is 84°, 96°, 54° and 107°, respectively. This is in agreement with the literature. Further work will be done with the focus on the nature of the surface chemical properties, such as the elemental composition of the surface and stability, which can be analyzed by X-ray photoelectron spectroscopy (XPS).
Nanocrystalline diamond layers and their variations in different surface terminations provide versatile possibilities for their applications in biological system. Different surface terminations can be chosen in the adjustment of the types of cells. Cells can selectively adhere or not adhere to the functionalized surface according to the specific demand. For example, it is reported that when osteoblasts were placed on the alternatively terminated diamond surfaces, i.e., terminated with hydrogen and oxygen at different zones, they preferred to adhere to the oxygenated site.\textsuperscript{45}

Among all the three surface terminations as mentioned above, hydrogen-terminated nanocrystalline diamond layers receive particular attention due to their distinctive and interesting properties. For example, they are electrical conductive, when exposed to air; extremely low friction coefficient is measured between two such sliding surfaces. In this thesis, it is explored that on hydrogenated diamond surfaces the masking nanoscopic interfacial water layers are with high organization and stability, which would play a key role in these extraordinary properties. This will be described in chapter 3. This discovery finally brings novel insight into the biological applications of nanocrystalline diamond layers, which will be presented in chapter 4.
Materials: nanocrystalline diamond layers
3 Interface between materials and biological systems: nanoscopic interfacial water layers

In biomaterials science, an interface bridges the materials and biological systems, and has been intensively studied. Events occurring at the interface determine the success or failure of the biomaterials’ applications. Regarding the interfacial systems, the most fundamental and challenging ones are nanoscopic interfacial water layers, which prevail on all the solid surfaces and have been found to play essential roles in numerous biological activities.\textsuperscript{21-24}

Since interfacial water layers are sensitive to observation, it is a great challenge to reveal their spatial arrangement, understand and manipulate them at the nanoscale. In this chapter, four different approaches are presented to probe the interfacial water layers experimentally and investigate their properties systematically. At first, section 3.1 gives an introduction to the interfacial water layers, regarding their molecular structure and physical properties. Next, section 3.2 deals with the analysis of the molecular organization of interfacial water layers on hydrogenated nanocrystalline diamond surfaces by examining the effect of electrical conductivity. In a previous study, a concept of dual technique has been introduced to probe the interfacial water layers: one tool (low-level laser light) is only to induce a change into the system; simultaneously the other independent tool monitors the responses of the system induced by the laser light.\textsuperscript{46} From section 3.3 to 3.5, three variations of the dual technique are reported: in combination with the low-level laser light, atomic force acoustic microscopy (AFAM), quartz crystal microbalance (QCM) and soft X-ray absorption spectroscopy (XAS) are applied respectively. Substrates with different surface polarities are used. These experiments result in an increasingly clear picture of interfacial water layers on model hydrophobic and hydrophilic surfaces in general and hydrogenated nanocrystalline diamond surfaces in particular, both in air and under water. The synoptic perspective emerging from the three different dual analyses of
interfacial water layers is, however, not only the information of their physicochemical properties, but also the knowledge of how to practically influence and modulate them.

3.1 Introduction to nanoscopic interfacial water layers

As early as 1971 Szent Györgyi provided a list of fundamental functions of interfacial water layers in biology in a visionary paper. It includes, but is not limited to, myosin-actin function, protein folding, shear force in blood, ion transport and origin of life. Further researches strengthened the biological implications of interfacial water layers ranging from acting as informational blueprints during the first contact events in the cell-material and the cell-cell contacts to slaving macromolecules activities. The potential of interfacial water layers is enormous, however, many of their anticipated implications have not been verified because of the experimental complexity in probing their structure and properties.

Over the years different techniques have been used to study interfacial water layers. Examples of these techniques are neutron scattering, X-ray diffraction, sum-frequency vibrational spectroscopy, ultrafast electron crystallography and atomic force microscopy. These experiments provide valuable information on interfacial water layers. However, due to their sensitivity to observation, information extracted from probing interfacial water layers with one single tool can be critical: important data, such as dynamic structure, interfacial residence time, or bond stability might return in a distorted form during “looking at” it. Hence, the concept of dual technique has been introduced. In a previous study, near-field scanning optical microscopy (NSOM) has been applied to monitor the profile of interfacial water layers on the surface of a transparent polymer film upon 670 nm laser irradiation at an intensity of 1000 W·m⁻². Here it is essential to discriminate the interaction of 670
nm laser light radiation with liquid water and with interfacial water layers. For water molecules there are three basic modes of vibrations: symmetric stretching ($v_1$), asymmetric stretching ($v_3$) and bending ($v_2$), as shown in Figure 3.1.1a. The most intense vibrations occurs when the wavelength of the radiation is matching the specific energy required for these motions, which can be recognized as peaks in the absorption spectrum of liquid water (Figure 3.1.1b). The bending vibrations contribute to an absorption peak around 6000 nm. The symmetric and asymmetric stretching have a similar energy requirement, forming a large absorption peak at around 3000 nm. Harmonics of the bending and stretching vibrations can be recognized as the subtle shoulders in the visible and near-infrared range of the absorption spectrum of liquid water (Figure 3.1.1c). In other words, liquid water shows an extremely weak absorption for visible light, such as 670 nm, primarily due to the combination of harmonics and fundamentals of stretching and bending modes.$^{57,58}$ The interaction of visible light with interfacial water layers, such as reflection/absorption, remains unknown in the literature. In addition, it is important to distinguish the low-level light used here from high intensity light used in other cases. For instance, light at an intensity of $1 \times 10^{13}$ W·m$^{-2}$ was applied in sum-frequency vibrational spectroscopy to probe interfacial water layers at solid-water interfaces. These experimental techniques and molecular dynamics simulations$^{59-61}$ have pointed out that the structure and properties of interfacial water layers are quite different from those of bulk water. A full picture of interfacial water layers, however, has not yet been achieved.
Figure 3.1.1 (a) The three vibrational modes of the water molecule and their fundamental frequencies in liquid water. (b) Absorption spectrum of liquid water. (c) Absorption spectrum of liquid water in the visible and infrared region.\textsuperscript{58}

The molecular structure of interfacial water layers on a substrate is determined by forces of orientation at the interface, the net charge density, which establishes the hydrophilic or hydrophobic character of the substrate,\textsuperscript{52} and the geometry of the
The interface-induced water layers undergo structural changes such as layering effect, a reduction in the mobility of water molecules in the first one to three layers. Various experimental observations and computer simulations have presented clear evidence that nanoscopic interfacial water layers are ordered on the nanometer scale on surfaces of different types (hydrophilic or hydrophobic). It is reported that the ordering persists for three to four layers (~1nm) on a hydrophilic surface (chlorine terminated silicon (111) substrate). Similarly, it is found that interfacial water on KH2PO4 crystal substrate presents the ordering in the first four layers, where the first two layers behave most ordered and are strongly bound to the surface and the next two layers are more diffuse and show minor vertical and lateral ordering. Importantly, these ordered interfacial water molecules do not form the typical ice structure (cubic or hexagonal), but are involved in many different forms of hydrogen bonding networks. A recent study presents that the hydrated water in a polymer, poly(2-methoxyethyl acrylate), can be classified into three types on the basis of the equilibrium water content and the enthalpy changes due to the phase transition observed using differential scanning calorimetry. The first type is the monolayer of water molecules that are tightly bound to the polymer surface. The second type is the layers that are weakly bound to the surface, which is called intermediate water. The third type is called free water, which is unable to shield the polymer surface or the tightly bound water, and freely exchanges with bulk water. It is hypothesized that when the intermediate water becomes sufficiently thick, it prevents the proteins and cells from directly contacting the polymer surface or the tightly bound water, and has an important role in the biocompatibility of polymers.

It is well-known that most fundamental physical properties of a material are changed if the geometry size in at least one dimension is reduced to a critical value (nanometer scale). Due to surface and interface interactions, nanoscopic interfacial water layers exhibit unique physical properties that are distinctly different from those of bulk water. Many experiments and computer simulations have shown that the interfacial...
water layers, which are confined between the hydrophilic surfaces at gaps below a few nanometers, present a dramatic increase in their viscosity.\textsuperscript{55,63,67-69} For instance, a recent atomic force microscopy study reports that the interfacial water layers between an oxide-terminated tungsten tip and the silica surface, both under ambient conditions and under water, present a viscosity of 6 to 7 orders of magnitude greater than that of bulk water.\textsuperscript{67} Moreover, another group shows that the viscous shear forces, reflected as viscosities, can be orders of magnitude higher than in bulk water if the subnanometer confining surfaces are hydrophilic, in addition they increase when the distance of the confining surfaces decreases. The viscous shear forces greatly decrease when the confining surfaces are increasingly hydrophobic.\textsuperscript{63} There is no observation of viscosity increase for a hydrophobic surface (graphite).\textsuperscript{55} In addition, theoretical studies and molecular dynamic simulations based on experiments indicate that the density of interfacial water layers is higher than that of bulk water.\textsuperscript{60,70-72} For instance, molecular dynamics simulations derived from X-ray and neutron solution scattering data show that the first monolayer of interfacial water (the 3-Å-thick first hydration layer) on the surface of lysozyme is 15% denser than bulk water. The density increase is the result of the geometry contribution and modifications of the water structure and dynamics, involving the shortening of the average water – water molecule distance and an increase in the coordination number.\textsuperscript{57}

### 3.2 Implication of nanoscopic interfacial water layers in effect of electrical conductivity on hydrogenated diamond

Intrinsic diamond with a wide band gap of 5.47 eV and an electrical breakdown field of $2 \times 10^7$ V cm$^{-1}$ is an excellent insulator.\textsuperscript{30,31} However, in 1989 Landstrass and Ravi first reported their experimental discovery that the hydrogenated CVD diamond layers
displayed a fairly low resistivity of the order of $10^6 \, \Omega \, \text{cm}$ at room temperature.\textsuperscript{73} Although the detailed mechanism of this effect is until now not yet understood, recent experimental evidence showed that it was not only postulated by the hydrogen termination, but also strongly affected by the nanoscopic interfacial water layers adsorbed on the surface of the hydrogenated diamond.\textsuperscript{74} Considering the properties that the diamond surface is chemically inert and shows extremely low oxidative tendency,\textsuperscript{75} hydrogenated diamond surface provides a unique platform to investigate interfacial water layers both in air and under water.\textsuperscript{76-80} In this section, hydrogenated nanocrystalline diamond surfaces are used to extract information on the properties of interfacial water layers by measuring the resistances in air and under water. Importantly the focus of my electrical conductivity experiments is restricted to the contact zone between the electrodes and the diamond substrates.

### 3.2.1 Introduction to electrical conductivity of hydrogenated diamond

Over 20 years, many research efforts have been invested in explaining the effect of electrical conductivity of hydrogenated diamond. In the first discovery report in 1989 by Landstrass and Ravi, the electrical conductivity of \textit{as-grown} CVD diamond layers grown in a hydrogen rich plasma is supposed due to the hydrogen passivation of the traps in the layers through the experimental observation that heating the layers leads to an increase in the resistivity by up to six orders of magnitude, as annealing causes dehydrogenation and may result in the electrical activation of the deep traps.\textsuperscript{73} Later, various experiments have been carried out by different research groups in order to elucidate the role of hydrogen in this electrical conductivity effect. Several different models have been proposed. For instance, it was suggested that the high conductivity is attributed to the shallow acceptor levels induced by the incorporated hydrogen in
the subsurface region with a depth of ~ 20 nm. In addition, another group proposed that the hydrogen-induced acceptors in the diamond are buried below the surface by a separation layer with a thickness of 30-50 nm.

Recently, it has been found that the nanoscopic interfacial water layers adsorbed on the surface of hydrogenated diamond is essential for the conductivity by performing experiments both in ultra-high vacuum and in air. The electrochemical model, specifically, the surface transfer doping model (as illustrated in Figure 3.2.1) is proposed.

![Figure 3.2.1](image)

**Figure 3.2.1** The transfer doping model. Top: Hydrogenated diamond surface with adsorbed interfacial water layer from air. Bottom: Evolution of band bending during the electron transfer process at the interface.

In detail, the interfacial water layers formed on the surface of hydrogenated diamond provides an electron system and act as a surface acceptor layer for diamond. The redox reaction \(2H_3O^+ + 2e^- \leftrightarrow H_2 + 2H_2O\), which is driven by the difference in the chemical potential of electrons in the liquid phase \(\mu_e\) and in the diamond (Fermi level \(E_F\)), governs the electron exchange from the diamond to the interfacial water.
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layer adsorbed from air. When $\mu_e$ is below $E_F$, electrons are transferred from the valence band in the diamond subsurface (donor) into the water layer (acceptor medium). This results in the formation of positive charge carriers (holes) in the diamond subsurface, which establishing a current parallel to the diamond surface. The associated space charge induces a potential (surface band bending) that raises $\mu_e$ until the thermodynamic equilibrium state ($\mu_e = E_F$). The surface transfer doping of diamond is regarded as an unconventional doping for semiconductor. Similar mechanism has recently been demonstrated for fullerene serving as the surface acceptors on hydrogen terminated diamond. The reason that diamond is particularly susceptible to surface transfer doping is regarded mainly due to that the hydrogen termination of the surface bond can reduce the electron affinity to the lowest value of all semiconductors, and it is possible to intimate contact with surface dopants because of no solid oxide on the diamond surface.

Later many experiments have been carried out under different conditions by different research groups. Some experimental observations can be entirely or partly explained in the frame of the transfer doping model. For example, the conductivity measurements were made by exposure to atmospheric vapors with different pH values (air, HCl and NH$_3$). The authors observed that the conductivity decreased with the increasing pH. This result can be explained with the transfer doping model. When the adsorbed film is more acidic, the equilibrium chemical potential $\mu_e$ is reduced, which facilitates the electrons transfer from the diamond to the film, leaving a positive charge layer (hole accumulation layer) in the diamond and giving rise to conductivity. In addition, the transport model is discussed by investigating the electrical properties of both hydrogenated CVD diamond and natural type IIa (100) diamond with Hall effect and conductivity experiments in the low temperature regime 0.34 – 350 K. The results suggested that a hole accumulation layer at the valence band edge (localized and extended states are present), is generated by the diffusion of valence band electrons into the surface adsorbates acting as an electron sink (“transfer
doping”). The propagation of holes is dominated by the electronic states, which are affected by the non-perfect hydrogen termination. Another group investigated the loss and recovery of surface conductivity of hydrogenated diamond upon annealing in vacuum (temperatures 600-900 °C) and exposure to different atmospheres (ozone-oxygen mixtures). The transfer doping model is able to account for the reversible loss and recovery of conductivity for annealing in vacuum below temperatures about 190 °C, and the irretrievable loss of surface conductivity after oxidation and for annealing temperatures above about 750 °C. However, it cannot account for the loss of surface conductivity after annealing in the temperature range (250 – 700 °C) and its recovery after UV illumination. Finally, the authors postulated that oxygen-related catalytic centers at the surface are necessary for the formation of the hole accumulation layer.

Moreover, some experiments did not follow the predictions by the transfer doping model. For example, the surface conductivity was measured by immersing the hydrogenated diamond layers in aqueous electrolyte with variable pH values when a gate electrode was used to control the diamond/electrolyte interfacial potential. Most experimental results of the hydrogenated diamond layers in aqueous solution were reported to be in clear disagreement with the transfer doping model. The authors discussed the energy band diagrams of the hydrogenated diamond/air interface and the hydrogenated diamond/aqueous electrolyte interface (see Figure 3.2.2). At the diamond/air interface, the electron affinity $\chi = -1.3$ eV and at the diamond/electrolyte interface $\chi = -1.0$ eV. The position of the valence band maximum at the surface ($E_{vs}$) is determined by the value of $\chi$. In the case of panel a, it is assumed that the thermodynamic equilibrium is reached when Fermi level $E_F$ is equal to electrochemical potential of electrons $\mu_e$ by the charge transfer across the interface (transfer doping model). In the case of panel b, the potential at the diamond/electrolyte interface is determined by the active control with potentiostat, which forces the equilibrium by fixing the potential drop between $E_F$ and the level of
reference electrode ($\mu_{\text{REF}}$). It is therefore proposed that the almost ideally polarizable diamond/aqueous electrolyte interface allows for the capacitive charging of the surface, that is, no charge transfer across the interface.\textsuperscript{88}

![Energy band diagrams](image)

**Figure 3.2.2** Schematic energy band diagrams of hydrogenated diamond/air interface (a) and hydrogenated diamond/aqueous electrolyte interface (b).\textsuperscript{88}

In the literature, other possible mechanisms of the electrical conductivity of hydrogenated diamond have been also proposed. For instance, it has been hypothesized that at least part of the electrical current flowing at or close to a hydrogenated diamond surface is carried by protons.\textsuperscript{89} Noting that the conductivity of hydrogenated diamond is a complicated and sophisticated issue, the understanding of this phenomenon requires the knowledge of surface science, semiconductor physics and electrochemistry.\textsuperscript{90} Any experimental details such as experimental setup, choice of diamond can alter the interpretation of the results.\textsuperscript{88,91} Although there is still much controversial in this issue, the present understanding, is that the interfacial water layers on hydrogenated diamond surface is one key factor in the electrical conductivity effect.
3.2.2 Nanoscopic interfacial water layers on hydrogenated diamond at solid-air interface

The hydrogenated nanocrystalline diamond layers, used to examine the interfacial water layers, were deposited on silicon wafers and with the thickness of 1 µm, average grain size of 15 nm and root mean square roughness of 18 nm. The resistance measurements were carried out under ambient conditions using a digital multimeter (Voltcraft, M-3860M, Hirschau, Germany, maximum range 40 MΩ) and validated by the use of an integrating digital multimeter (PREMA, 316, Mainz, Germany). Spring-loaded platinum electrodes (purity 99.99%) were applied in a two-point configuration and acting as Schottky contacts.

At first, the resistances of the samples on both sides (points 1-2 and points 3-4 in Figure 3.2.3) as well as across the diamond-silicon junction (points 1-3 and points 4-2 in Figure 3.2.3) were measured to understand the electrical conductivity effect of the hydrogenated diamond. The experiments were performed at a temperature of 25 °C and relative humidity of 55% in dim light. The applied voltage was 109 mV. The following values were obtained: \( R_{12} = 1.186 \text{ MΩ} \), \( R_{13} = 0.454 \text{ MΩ} \), \( R_{34} = 0.020 \text{ MΩ} \) and \( R_{42} = 0.730 \text{ MΩ} \). Values were regarded as stable when there was no change during an observational period of 30 min. If we name the sum of the latter three resistance values as \( R_x \), we obtain:

\[
R_x = R_{13} + R_{34} + R_{42} = 0.454 \text{ MΩ} + 0.020 \text{ MΩ} + 0.730 \text{ MΩ} = 1.204 \text{ MΩ}
\]

From the relation that \( R_x \) is practically equal to \( R_{12} \), it was noted that the hydrogenated diamond samples were bulk conductive. Considering that the electrical conductivity effect was not observed on the non-hydrogen terminated diamond with the same surface profile and the same CVD-history, the possibility of an intrinsic grain boundary conductivity in general and \( sp^2 \) mediated conductivity in particular\(^92\) can be excluded.\(^78\)

28
In a next step, the influence of humidity on the electrical conductivity effect of hydrogenated diamond was checked since the presence of interfacial water layers is a key element in this phenomenon. The resistance was monitored before, during and after exposure of the diamond surfaces to the increased humidity conditions, for instance, by breathing onto the samples. At normal relative humidity, the macroscopic water vapor films vanish within seconds on hydrophobic substrates, in contrast to the nanoscopic interfacial water layers, which are invisible for naked eyes but masking the substrates. The resistance value $R_{12}$ increased upon breathing in a time on the order of one second from 1.186 MΩ to a maximum between 1.985 and 2.010 MΩ, and returned with a time constant of approximately 2 min to the initial value. The peak value depended on the performance of the experimenter. Alternatively, the impact of humidity on the electrical conductivity effect was demonstrated by holding a sterile tissue wetted with ultrapure water (conductivity 5.5 μS m⁻¹) close to the surface of the samples, thus excluding the possibility that the effect was caused by expired carbon dioxide or by an increase in temperature associated with the process of expiration. Control experiments of exposure to nitrogen gas were done and showed no influence on the electrical conductivity effect.

In addition, the resistances of hydrogenated diamond were recorded in corresponding to humidity changes in a time-dependent manner. The diamond sample was placed into a partially closed black box with a sterile tissue wetted with ultrapure water
nearby the electrodes, inducing slowly increased humidity levels. When the saturated humidity was reached, reflected as the stable value in resistance, the wetted tissue was removed from the box. Figure 3.2.4 shows that the resistance of hydrogenated diamond increased with increased humidity levels and the effect was reversible.

![Graph showing resistance over time](image)

**Figure 3.2.4** Resistance of hydrogenated diamond when exposed to different humidity levels at room temperature.

Furthermore, it is well-known that the properties of CVD nanocrystalline diamond are strongly influenced by grain boundaries, which usually consist of hydrogen and considerable amount of non-diamond carbon, such as $sp^2$ graphite or $sp^2$ and $sp^3$ hybridized carbon bonds. In the literature it has been reported that the effect of grain boundaries on the electrical properties of hydrogenated CVD nanocrystalline diamond, and found that the electrical properties are determined by the presence of the adsorbed interfacial water on the grain extern surfaces and by the local modification of the inter-grain material in which the conductivity may due to variable range hopping in band tails. In order to exclude possible confusion that might arise from synthetic diamond layers, a natural diamond single crystal cube was treated with hydrogen plasma and used to examine the electrical conductivity effect by measuring...
resistances in response to various different levels of humidity. The diamond sample was put into a partially closed chamber with several ultrapure water reservoirs to increase the humidity. A hygrometer was placed near the sample in the chamber to real-time monitor the relative humidity values. The multimeter and platinum electrodes applied previously on the synthetic diamond layers were used here for resistance measurements. The observed dependence of the resistance on relative humidity is consistent with that for hydrogenated CVD diamond layers. For instance, the resistance value was obtained from 112 kΩ at 41% relative humidity to 260 kΩ at 78.9% relative humidity.

In order to understand the observed relationship between the resistance and relative humidity (i.e., an increase in resistance with an increase in relative humidity), we start with recapitulating the currently most accepted model of the electrical conductivity of hydrogenated diamond, namely the surface transfer doping model (as described in section 3.2.2). From this model, one may expect that an increase in the capacity of the acceptor medium for electrons, realized by an increased thickness of the water layer on the top of the diamond surface, should be accompanied by an increase in electrical conductivity, caused by an increase in the number of positive charge carries in the diamond subsurface. However, our results cannot explain the electrical conductivity phenomenon in terms of the transfer doping model: an increased thickness of the water layer (higher relative humidity levels) caused a decrease in electrical conductivity (higher resistance values).\textsuperscript{76,77}

Noting that the water film adsorbed on a hydrogenated diamond surface is a prerequisite for the electrical conductivity effect, the basic understanding of the physicochemical properties of the interfacial water layers on hydrogenated diamond as well as other model surfaces is essential and in the focus of the investigations. Intrinsic to hydrogenated diamond is the triple coincidence: solid surface, pronounced hydrophobicity and unilateral affinity to water molecules due to the hydrogen atoms. Understanding this aspect is essential for a comprehensive analysis of the electrical
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conductivity effect. Previously, several experiments have been done to detect interfacial water layers on hydrophobic surfaces in air and under water. NSOM experiments show that ordered interfacial water layers are formed on a moderately hydrophobic polymer surface in air. The molecular order is induced basically by the unilateral restriction in the mobility of the water molecules (one degree of freedom less compared to bulk water molecules). Drop evaporation experiments indicate that ordered interfacial water layers prevail on the hydrophobic polystyrene surfaces under water. The ordering of the interfacial water layers is exposed by their spontaneous response (depletion) to the moderately intense 670 nm laser light, a wavelength that is practically not absorbed by normal bulk water. The depletion effect has been observed on hydrophobic surfaces. In the case of hydrogenated diamond surface (hydrophobic), the order of the interfacial water molecules is not only imposed by the spatial restriction by the solid surface but also chemically induced by the polarization of at least one layer of water molecules, mediated by the hydrogen atoms on the diamond surface. This picture may suggest that the stability of ordered interfacial water layers on hydrogenated diamond is superior to that on non-hydrogenated substrates. AFAM experiments proved this hypothesis, which will be described in section 3.3.

As will be discussed, the implication of the interfacial water layers in the electrical conductivity effect can be in principle indirect or direct. In the indirect mode, it facilitates the liberation/transport of holes in the diamond sample. In the direct mode, electrical conductivity could be related to the presence of excess protons, in accord with the high mobility of excess protons in water. Proton transfer depends on the water structure, which is probably highly ordered on hydrogenated diamond surface. According to Grotthuss mechanism, a proton is hopping from a hydronium ion (H$_3$O$^+$) to a neighboring water molecule. For the proton transfer, it is postulated that the water molecule closest to the H$_3$O$^+$, i.e., shortest oxygen distance (maximum order), is the most likely candidate to which the proton can be transferred.
Although there are uncertainties about the structural properties of the interfacial water layers on hydrogenated diamond surface, such as thickness and stability, which cannot be clarified from the electrical conductivity experiments, it is plausible to think that the order and stability will decrease with the increasing distance from the polarizing source (hydrogenated diamond surface). Taking the above-mentioned points into consideration, the picture of coexistence of highly ordered and less ordered water molecules on hydrogenated diamond surface can be drawn, as showed in Figure 3.2.5.

Thus, it can be expected that the increased relative humidity may disturb the order of interfacial water layers by excess of less ordered water molecules, resulting in a decrease in electrical conductivity. This is consistent with our experimental observations.\textsuperscript{76,98}

\textbf{Figure 3.2.5} Schematic side view of hydrogenated diamond surface, including the well ordered and less ordered interfacial water layers. The order decreases with the increasing distance from the diamond surface. Likewise, it decreases when the thickness of the “bulk water” phase increases.
3.2.3 Nanoscopic interfacial water layers on hydrogenated diamond at solid-water interface

With the purpose to understand the interfacial water layers at solid-water interface, the electrical conductivity experiments on hydrogenated nanocrystalline diamond were carried out under water (i.e. in water drops). The same measurement devices described in section 3.2.1, were used. The temperature and relative humidity were 24 °C and 48%, respectively. Two drops of 20 µl of ultrapure water were applied on the surface of hydrogenated diamond samples. As showed in Figure 3.2.6, the positions of the platinum electrodes are marked with numbers. The resistances between the position 1-2 (air-air), 1-3 (air-water), 4-2 (water-air) and 4-3 (water-water) were measured, respectively. Anode was in position 1 and 4. Cathode was in position 2 and 3. The distance between the two electrodes was 1.5 cm.

The following representative resistance values were obtained: $R_{12} = 1.3 \, \text{MΩ}$, $R_{13} = 5.63 \, \text{MΩ}$, $R_{42} = 21.29 \, \text{MΩ}$ and $R_{43} > 40 \, \text{MΩ}$. The resistance value $R_{12}$ (air-air) is in good agreement with the previous measurements. Obviously the resistance values measured with at least one electrode under water are much higher than those with both electrodes on dry hydrogenated diamond surface. In view of the previous experimental observation (increased relative humidity caused an increase in resistance) and its explanation, as described in section 3.2.1, it is possible to explain this phenomenon in a similar way: bulk water molecules in the drop may reduce the order and stability of the interfacial water molecules on hydrogenated diamond surface. This would disturb the chain of proton hopping, thereby resulting in a decrease in electrical conductivity (an increase in resistance).
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**Figure 3.2.6** Two 20 µl water drops on hydrogenated nanocrystalline diamond surface. The positions of electrodes (1, 2 on dry substrate; 3, 4 under water) are marked on the sample. Anode and cathode are also labeled.

The asymmetric resistance values between $R_{13}$ (cathode under water) and $R_{42}$ (anode under water) guide us to take a closer look at the specific situations between different electrodes and hydrogenated diamond surface. Up to here, the understanding of the interfacial water layers on hydrogenated diamond surface is the coexistence of highly ordered water molecules polarized from the hydrogen atoms on the diamond surface and less ordered water molecules with increasing distance from the hydrogenated diamond surface. Now we concentrate on the interspace between the electrodes and hydrogenated diamond surface and realize that in the case of the cathode the interconnecting interfacial water molecules are consistently polarized in one direction from both sides, as showed in Figure 3.2.7. Thus, the interfacial water molecules aligned between cathode and hydrogen atoms are more ordered compared to those between anode and hydrogen atoms. In other words, it could be probably more difficult for bulk water molecules to reduce the order of the interfacial water molecules when cathode is under water, compared to the situation when the anode is under water. This could explain why the resistance value $R_{13}$ (cathode under water) is relatively smaller than $R_{42}$ (anode under water). The relatively high resistance value $R_{43}$ (both electrodes under water) can also be explained with this principle:
unorganized bulk water molecules reduce the order of interfacial water molecules on both sides (cathode and anode), thereby causing a substantial reduction in electrical conductivity.

One can also apply the energy band diagram (Figure 3.2.2 b, page 27) to discuss the electrical conductivity of hydrogenated diamond immersed in water. For instance, when the applied potential on the diamond surface is positive, the position of $E_F$ will be driven below the valence band maximum, the holes will accumulate at the diamond/aqueous interface. A larger applied potential would lead to further band bending and increase the accumulation of the holes. However, when the applied potential on the diamond surface is negative, $E_F$ would be pushed eventually above the valence band maximum and the conductivity will finally disappear. However, in order to experimentally prove this, one needs to optimize the experimental setup and include the possibility of obtaining the conductivity at various applied potential, as reported.\textsuperscript{88}

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{schematic_side_view_diamond_surface.png}
\caption{Schematic side view of hydrogenated diamond surface in the resistance measurement. Highly polarized water molecules chains are formed between cathode and hydrogen atoms on the diamond surface; less ordered and/or stable chains are formed between anode and hydrogen atoms on the diamond surface.}
\end{figure}
In the literature, it is reported that under ambient condition the conductivity of hydrogenated diamond is of the order of $10^{-4}$ to $10^{-5}$ $\Omega^{-1}$, and the sheet carrier density is in the range $10^{12} - 10^{13}$ cm$^{-3}$ with a carrier mobility of $10 - 100$ cm$^2$ V$^{-1}$ s$^{-1}$.\textsuperscript{74,82,88} Due to the limitation of my conductivity experiments, such as the undefined geometry of the contact zone between the electrode and the hydrogenated diamond surface, and the undefined pathway of the current, there is no access to obtain the detailed information about the electrical properties of hydrogenated diamond, such as sheet resistivity. Moreover, several different groups has investigated the influence of humidity\textsuperscript{99} or aqueous on the electrical conductivity of hydrogenated diamond (see section 3.2.1).\textsuperscript{88} A fundamental difference between the reported experiments and my experiments is the focus area. In their paper, the focus is the area between the electrodes, whereas in my thesis the focus is the contact zone between the electrode and the substrate. In the reported paper, Au or Ti/Au contacts are usually deposited by electron-beam evaporation,\textsuperscript{87,98} and the van der Pauw technique, or Lampard-Thompson’s theorem can be used to evaluate the sheet conductivity. In my thesis, the resistances are measured using two-point method where the electrodes are in direct contact with the hydrogenated diamond surface. Therefore, one can get access to the information of the interfacial water at the electrode/diamond interface, the focus of my conductivity experiments. The electrical conductivity of hydrogenated diamond is a complicated issue and regarded as an “interdisciplinary research topic”.\textsuperscript{90} There is a lot of open questions, such as the detailed mechanism, the role of interfacial water on energy band bending, the stability of interfacial water at the electrode/diamond interface in air and in liquid water. Therefore there is a lot of further research to be done.

In summary, the electrical conductivity experiments of hydrogenated nanocrystalline diamond that were carried out under water (i.e., in water drops) indicate that ordered interfacial water layers prevail subaquatically, and the order of interfacial water molecules is reduced by the additional unorganized bulk water molecules. The results
confirm the picture of the interfacial water layers on hydrogenated diamond surfaces extracted from studying the solid-air interface in the electrical conductivity effect (section 3.2.2). The electrical conductivity of hydrogenated diamond is puzzling researchers since more than 20 years. Current ideas for the surface-conducting diamond could be expanded to include the interfacial water structure, presumably affecting both the interspace between electrodes and diamond and in the superficial space between the electrodes; doing so could enhance the predictive capabilities of emerging models. Whereas this was beyond the scope of the thesis, it is clear that further research on the mechanism of the electrical conductive of hydrogenated diamond is necessary.

3.3 Probing nanoscopic interfacial water layers by AFAM & laser light

Atomic force microscopy (AFM)\textsuperscript{54,55} and the related techniques, such as surface force apparatus (SFA),\textsuperscript{100} interfacial force microscopy (IFM)\textsuperscript{67} and friction force microscope (FFM)\textsuperscript{68} have been reported to probe interfacial water layers at solid-air interface. Important physical properties, such as viscosity and density could be acquired from these studies. The reason for using AFM-based techniques to study interfacial water layers is that they can be operated under ambient conditions, where interfacial water layers are always present. This can be seen from the AFM working principles: the interatomic force, either attractive or repulsive van der Waals force between tip and sample causes deflection of the cantilever, which will reflect the topography of the sample. The role of interfacial water layers in AFM measurements is also highlighted in a recent all-atom molecular dynamic stimulation study, which suggests to use AFM to study interfacial water layers.\textsuperscript{101}
In this section, atomic force acoustic microscopy (AFAM), a new AFM based measuring technique which allows quantitative and qualitative measurements of the local surface elastic properties of many materials, is embed into the dual technique. That means, a moderately intense 670 nm laser light is used to modulate the structure of interfacial water layers that are prevailing at solid-air interface, as presented in the previous study, and simultaneously the changes at the AFM tip-sample interface are detected by monitoring the altered resonance frequency and amplitude of the cantilever. The AFAM based dual technique offers reliable and instructive information about interfacial water layers on a variety of substrates.

At an earlier time, the combination of NSOM and 670 nm laser light has been successfully used to probe the interfacial water layers. However, due to the limitation of the experimental setup, only optical transparent samples could be employed. The advantage of the AFAM based dual technique is that it allows to detect interfacial water layers practically on all smooth materials surfaces. In this study, hydrophobic polystyrene and hydrophilic silicon were chosen as model substrates to investigate interfacial water layers on surfaces of non-polar and polar materials. In a next step, a hydrogenated nanocrystalline diamond sample was applied. On the surface of hydrogenated diamond, the interfacial water molecules are not only imposed by the spatial restriction by the solid surface but also chemically mediated by the hydrogen atoms on the diamond surfaces, as indicated by the electrical conductivity experiments in section 3.2. This picture has been recently reinforced. For comparison, a non-hydrogenated nanocrystalline diamond sample with identical grain size and roughness was also used. Finally the experimental results may explain the mysterious phenomenon – the extremely low friction between two sliding hydrogenated carbon based materials surfaces such as hydrogenated diamond and hydrogenated diamond-like carbon.
3.3.1 Atomic force acoustic microscopy (AFAM)

The principle of AFAM has been developed by Rabe and Arnold in 1994. The basic idea is to excite and measure the AFM cantilever flexural vibrations when the tip is in contact with the sample. In AFAM, the sample of interest is mounted on an ultrasonic transducer and the longitudinal ultrasonic waves are injected into the sample, which is reflected at the surface of the sample as vertical displacement. When the sample is brought in contact with the AFM tip at an applied force, a specific contact resonance frequency is obtained by recording the vibrational amplitude of the AFM cantilever as a function of the corresponding ultrasonic excitation frequency. Thereby, the contact stiffness is extracted from the measurements. From the contact stiffness, the elastic properties of the sample, such as Young’s modulus can be calculated according to appropriate models, such as Hertzian contact mechanics.

The coupling between the AFM tip and the sample surface can be interpreted with a beam dynamics model using analytical or finite-element analysis approaches. Additionally, as the shape of the resonance curves is Lorentzian, the vibration dynamics can be approximated to that of a point-mass model (first mode approximation). In a simple term, the tip-sample interaction is described as the classical single spring constant, representing the system’s contact stiffness $k$, as illustrated in Figure 3.3.1. This approximation is valid when the applied force is much bigger than the attractive force but still smaller enough to avoid deformation of the sample.

![Figure 3.3.1](image)

Figure 3.3.1 Schematic drawing of the simplified approximation of the AFM tip and the sample surface interaction.
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Usually, the oscillation manner of AFM tip is expressed in terms of the quality factor ($Q$ factor), which determines the qualitative behavior of damped oscillators and can be calculated as follows:

$$Q = \frac{f}{\Delta f}$$

where $f$ is the resonance frequency, $\Delta f$ stands for half width of the resonance curve.

When AFAM is performed at resonance frequency, the resonance amplitude is proportional to the $Q$ factor and inversely proportional to the damping. As a general tendency, the resonance amplitude increases with increasing contact stiffness.108

3.3.2 AFAM on model hydrophobic and hydrophilic surfaces

Figure 3.3.2 shows an experimental setup of the AFAM based dual technique to measure the interfacial water layers between the AFM tip and the sample at a fixed position. AFAM (Fries Research & Technology) was employed to record the resonance spectra at room temperature with controlled relative humidity levels. A single crystal silicon tip was mounted on a soft cantilever (0.2 N·m$^{-1}$), applied with very small loading forces. The tip-sample interface was irradiated by a 670 nm laser (laser 2 in Figure 3.3.2) at a small angle of incidence of 11.5°, with a power of 0.8 mW, and a local intensity of 50 W·m$^{-2}$. It is worth mentioning that the irradiation at a local intensity of 50 W·m$^{-2}$ is very low. In the NSOM46 and drop evaporation93 studies, the 670 nm laser light used to modulate the interfacial water layers was at a local intensity of 1000 W·m$^{-2}$ (~ solar intensity). For comparison, we further calculate the intensity of a 1 mW laser pointer with a beam diameter of 1 mm and obtain a value of 1.3 kW·m$^{-2}$. 

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Figure 3.3.2 Experimental setup of AFAM based dual technique. The basic component of AFAM comprises AFM performing in contact mode on an ultrasonically excited sample. Laser 2 is applied to modulate the interfacial water layers between the AFM tip and the sample. Laser 1 does not illuminate the tip-sample interspace. Inset illustrates the interfacial water confined between the AFM tip and the sample surface.

A polystyrene sample (material from the cover of Petri dish) was used to explore the interfacial water layers on hydrophobic surface in air. The experiments were performed at a temperature of 24 °C and relative humidity of 61%. The applied loading force was 5 nN. Figure 3.3.3 presents the AFAM resonance spectra, without and with laser light irradiation. In order to analyze the damping at the tip-sample interface, the $Q$ factor was calculated without laser light (dim light $Q_D$) and with laser light ($Q_L$) irradiation after extracted from the Lorentzian fit to the measured data. The following values were obtained: $Q_D = 26.5$, $Q_L = 39.3$. From the inversely proportional relation between $Q$ factor and the damping, it was found that the damping at the tip-sample interface was decreased after laser light irradiation. Noticing that the interfacial water layers between the tip and the sample could be regarded as a damping element and thereby softening the interface contact, one can understand this effect in the following way: the laser light irradiation weakens the damping function of the interfacial water that behaves like a solid (organized)
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between the hydrophilic tip and the hydrophobic surface, leading to an increase in $Q$ factor. This is in agreement with the aforementioned experimental results: 670 nm laser irradiation can deplete the ordered interfacial water layers on hydrophobic surfaces.\textsuperscript{46,93}

Figure 3.3.3 AFAM resonance spectra on hydrophobic polystyrene surface without and with 670 nm laser irradiation.

Next, a silicon wafer sample (100) was used to explore the interfacial water layers on hydrophilic surface in air. The temperature and relative humidity was 24 °C and 61\%, respectively. The applied loading force was 10 nN. Figure 3.3.4 shows representative AFAM resonance spectra, without and with laser light irradiation.\textsuperscript{102} After calculation from the Lorentzian fit to the measured data, the following values of $Q$ factor without and with laser light irradiation were obtained: $Q_D = 34$, $Q_L = 24.5$. Interestingly, it was found that compared to hydrophobic surface, the laser light irradiation on hydrophilic surface had an opposite effect: the damping at the tip-sample interface was increased after laser light irradiation. This exposes that interfacial water layers on polar and non-polar surfaces have different properties.
From the definition of hydrophilic (water loving) and hydrophobic (water hating), one can reasonably assume that probably the interfacial water layers masking hydrophilic surface are thicker than those on hydrophobic surface are. In view of the reported AFM study, the water confined at the nanoscaled interfacial separation between the hydrophilic tip and the hydrophilic sample shows a viscosity of 6 to 7 orders-of-magnitude larger than that of bulk water under ambient conditions.\textsuperscript{67} Taken together with the experimental observation, i.e., an increase in the interface damping upon 670 nm laser irradiation, one could expect that the laser light irradiation at the interface reduced the viscosity of the interfacial water layers. Since viscosity means resistant to flow, a simple scenario that provides a consistent explanation for the notable result is proposed in the following: the laser light irradiation on hydrophilic surface caused an increased fluidity in the viscous interfacial water layers, therefore the AFM tip penetrated deeper and was trapped in the viscous interspace – reflected in an increased interface damping.

\textbf{Figure 3.3.4} AFAM resonance spectra on hydrophilic silicon surface without and with 670 nm laser irradiation.
In addition, it is worth mentioning that the 670 nm laser irradiation-caused shift in resonance frequency and amplitude is reversible. Moreover, this effect strongly depends on relative humidity. In this study, it was found that the laser light irradiation had no effects when the relative humidity is below 48%. Figure 3.3.5 shows the AFAM resonance spectra on the hydrophobic polystyrene surface without and with 670 nm laser irradiation at a relative humidity of 45%. This phenomenon excludes the possibility that the observed effects (shift in resonance frequency and amplitude) are from other sources, such as heating of the AFM tip and/or the substrate. In conclusion, AFAM based dual technique is used to show that 670 nm laser irradiation can deplete the ordered interfacial water layers on the hydrophobic surfaces and fluidize the viscous interfacial water layers between the hydrophilic surfaces under ambient conditions.

![AFAM resonance spectra on hydrophobic polystyrene surface without and with 670 nm laser irradiation, showing that the laser light irradiation had no effects when the relative humidity was 45%.](image)

**Figure 3.3.5** AFAM resonance spectra on hydrophobic polystyrene surface without and with 670 nm laser irradiation, showing that the laser light irradiation had no effects when the relative humidity was 45%.
3.3.3 AFAM on non-hydrogenated and hydrogenated nanocrystalline diamond surfaces

The interfacial water layers on hydrogenated diamond surface are highly ordered, especially for the very first few layers close to the surface; and these highly ordered layers are with high stability, due to the bond by the hydrogen atoms on the diamond surface. This has been reinforced recently.\textsuperscript{104} In this section, I explore the stability of the interfacial water layers on hydrogenated and non-hydrogenated nanocrystalline diamond by applying the established AFAM based dual technique.

The experimental setup was the same as illustrated in Figure 3.3.2. The experiments were carried out at a temperature of 24 °C and relative humidity of 30%. The nanocrystalline diamond samples were with the average grain size of 15 nm and root mean square roughness of 18 nm, for both non-hydrogenated and hydrogenated ones. A 670 nm laser beam (power 5 mW and local intensity 625 W·m\textsuperscript{-2}) was used to irradiate the AFM tip-sample interface.

For non-hydrogenated nanocrystalline diamond sample, AFAM resonance curves (Figure 3.3.6)\textsuperscript{103} show a typical response to the laser light irradiation on hydrophobic surface, see Figure 3.3.3. $Q$ factor was calculated after extracted from the Lorentzian fit to the measured data and it was found that $Q$ factor was changed from 14 to 16, which indicated that upon irradiation the depletion of the damping elements (interfacial water layers) occurred and the tip-sample contact became stiffer.
In a next step, the AFAM measurements on hydrogenated nanocrystalline diamond sample under the same conditions were performed. Figure 3.3.7 shows that the laser light irradiation had no influences in AFAM resonance frequency and amplitude in this case.\(^{103}\) This implies there was no change in the tip-sample interface with the laser light irradiation. Since the polarity difference between the hydrogenated and non-hydrogenated diamond surface can be neglected (contact angels are comparable), it can be assumed that equally thick water films on both diamond species. In this context, the result suggests that the interfacial water molecules on hydrogenated nanocrystalline surface are strongly bounded. This argument stems from the chemical affinity of carbon towards hydrogen: C – H bonding is covalent and very strong (stronger than C – C bonds). The consequence is a noticeable tendency to bind and polarize water molecules. In other words, it is associated with a more stable interfacial water layers compared to those on the non-hydrogenated diamond surface.
In addition, this result is crucial in understanding the role of hydrogen in controlling the surface properties of hydrogenated diamond and even other hydrogenated carbon based materials, especially the reported super low friction phenomenon between two hydrogenated carbon based materials surfaces. In general, friction is the consequence of a mixture of chemical, physical and mechanical interactions between two sliding surfaces. The simplest approach explains the super low friction between hydrogenated diamond surfaces as well as between hydrogenated diamond-like carbon surfaces as the manifestation of electrostatic repulsion between the hydrogenated sites in direct (so called dry) contact. Notably, under realistic experimental conditions the function of water has been intensively discussed at the macroscale but never at the nanoscale. Here the role of interfacial water layers in the friction issue will be discussed. Figure 3.3.8 provides the idealized presentation of the interfacial water layers at the interface of two non-hydrogenated (a) and hydrogenated (b) diamond surfaces, respectively. Whereas for non-hydrogenated diamonds relative linear movement of the slabs is associated with energy dissipation due to viscosity.
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effects caused by a mobile interfacial phase, for hydrogenated diamonds, water molecules anchored to the surfaces facilitate the relative movement of the slabs, in agreement with the super low friction observed experimentally.

Interestingly, this principle is coincidently similar with that of water molecules tenaciously attached to the zwitterionic polymer brushes used in the joint lubrication model.\textsuperscript{112} Moreover, it can be better understood that the practically frictionless water flow in certain carbon nanotubes\textsuperscript{113} by including the confined ordered interfacial water layers as a lubricate. In short, it has been demonstrated that the interfacial water layers are highly stabilized by the hydrogen atoms on the diamond surface and they play a key role in the physical and chemical interaction during sliding of two surfaces. Therefore, the results are promising in extending the current friction model\textsuperscript{114,115} under realistic conditions at the nanoscale.

\textbf{Figure 3.3.8} Artist’s view of ordered interfacial water layers between two diamond surfaces: (a) non-hydrogenated and (b) hydrogenated.
3.4 Probing nanoscopic interfacial water layers by QCM & laser light

The quartz crystal microbalance (QCM) is a high-resolution mass sensing technique with sensitivity at nanogram level and has been widely used in many fields such as surface chemistry, biochemistry and biomedical engineering to elucidate the solid-air and the solid-liquid interfacial phenomena, in particular for subtle objects.\textsuperscript{116-118}

In this section, I apply the dual technique concept with the combination of QCM and low-level laser light to probe interfacial water layers at solid-air as well as solid-liquid interface. In the experiments, a moderately intense laser light (670 nm, 633 nm) was used to irradiate interfacial water layers on model hydrophobic (polystyrene) and hydrophilic (gold) surfaces, in the meantime, the response of QCM on resonance frequency was monitored. The results provide detailed properties of interfacial water layers on model surfaces under ambient conditions, which are consistent with the previous NSOM\textsuperscript{46} and AFAM studies.\textsuperscript{102,103} One specific advantage of this technique is that it allows to investigate interfacial water layers both in air and under water. Moreover, it provides quantitative information of interfacial water layers, such as thickness.

3.4.1 Quartz crystal microbalance (QCM)

Basically, QCM is a mass loading sensor based on the piezoelectric effect. The heart of a QCM is a thin quartz crystal disk with gold electrodes on both surfaces performing shear oscillations parallel to its plane. An alternating electric field is applied to the quartz to induce an alternating expansion and contraction of the crystal lattice, as illustrated in Figure 3.4.1.
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Figure 3.4.1 (a) A typical QCM sensor with gold coating on the bottom (yellow) as well as the top of crystal (not shown here). The gold coating works as electrode and substrate. (b) A quartz crystal performs shear oscillations with alternating current applied on both sides.

QCM measures the mass of a material deposited on the quartz crystal surface according to the Sauerbrey equation,\(^\text{119}\) which relates the frequency change $\Delta f$ of the quartz crystal and the mass change $\Delta m$ of the layer adsorbed to the crystal surface:

$$
\Delta m = -C \frac{\Delta f}{n} A
$$

with $A$ is the surface area, $n$ is the actual overtone number of the oscillator circuit, $C$ is the mass sensitivity constant and given as follows:

$$
C = \frac{\nu \rho_Q}{2f^2}
$$

where $\nu = 3340 \text{ m s}^{-1}$ is the speed of sound in the quartz crystal, $\rho_Q = 2.651 \text{ g cm}^{-3}$ is the density of the quartz crystal, $f$ is the resonance frequency.

Importantly, the Sauerbrey equation is based on the following three assumptions: the mass of the adsorbed layer $\Delta m$ is relatively smaller than the mass of the quartz crystal; the adsorbed layer is rigidly bounded to the surface; and the adsorbed layer evenly spreads over the active crystal surface. This equation reveals that a decrease in mass
on the crystal surface will result in an increased resonance frequency linearly. In addition, quantitative information of the thin film tightly adsorbed on the crystal surface can be obtained. In this study, the thickness of nanoscopic interfacial water layers will be acquired.

3.4.2 QCM on model hydrophobic and hydrophilic surfaces

Figure 3.4.2 illustrates the experimental setup of QCM based dual technique to probe interfacial water layers in air and under water. A QCM (Q-Sensor D300, Sweden) was applied to monitor the resonance frequency upon laser light irradiation on different materials coated gold quartz crystal in air (temperature 23.8 °C, relative humidity 32.2%) and subaquatically (under a 3 mm column of ultra pure water). The sensor was mounted in a window chamber that permitted the irradiation of the sensor at an angle of 90° from the top, and 45° from the side. Two different wavelengths (670 nm, 633 nm) of laser light with the same power (5 mW) and the same spot diameter (~ 4 mm) were tested, respectively. Light intensity for laser beam incidences normal to the sensor surface (see laser position 1 in Figure 3.4.2) was as low as 400 W·m⁻².

In order to discriminate from possible thermal effect of the laser light irradiation on the one hand, and investigate the interaction of the photons with interfacial water molecules on the other hand, the sensor was irradiated at an incidence of 45° (see laser position 2 in Figure 3.4.2). Different laser polarization to the sensor plane was realized by rotating the laser housing around the beam path (parallel beam). Two different wavelengths (670 nm, 633 nm) of laser light with the same power (5 mW) and the same beam diameter (~ 1 mm) were tested, respectively.

The hydrophobic target surfaces were gold electrodes with spin-coated polystyrene¹²⁰ (water contact angle 94°). The hydrophilic target surfaces were gold electrodes (water contact angle 42°). The samples were cleaned with a solution of
boiling aqueous ammonia (30%), H$_2$O$_2$ (30%) and water (ratio 1:1:5), followed by rinsing in ultrapure water and drying under a nitrogen stream.

**Figure 3.4.2** Experimental setup of QCM based dual technique. The sensor is mounted in a window chamber, where it can be filled with water. The geometry of the chamber permits the laser light irradiation from the top (laser position 1) or at an angle of 45° to the surface of the sensor (laser position 2), both in air and under water.

Figure 3.4.3 shows a typical frequency change of the sensor in response to laser light irradiation when the beam is perpendicular to the sensor surface (laser position 1 in Figure 3.4.2). When the laser light was switched on, there was a spontaneous increase in the resonance frequency of the sensor, whereas when the laser light was switched off, a spontaneous decrease was observed and the frequency reverted to its initial value. This indicates that upon laser light irradiation, layers of rigid tightly packed thin film, in this case referring to the interfacial water layers that are adsorbed on the surface of the sensor crystal, are detached from the sensor surface, as illustrated in Figure 3.4.4. This effect is reversible.
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Figure 3.4.3 Resonance frequency change $\Delta f$ of the hydrophobic polystyrene coated quartz crystal when 633 nm laser light is switched on (off).

![Figure 3.4.3](image)

Figure 3.4.4 Schematic drawing illustrates that when the laser light is switched on, interfacial water layers are detached from the sensor surface.

![Figure 3.4.4](image)

As described in section 3.4.1, the Sauerbrey equation provides the possibility of calculating the thickness of the adsorbed interfacial water layers. In the experiments, the resonance frequency $f$ is 5 MHz and the overtone number $n$ is 7 (that is, the actual frequency of the oscillator is 35 MHz). In the derivation of the Sauerbrey equation, it is assumed that the mass change $\Delta m$ on the quartz crystal is evenly distributed over the complete active area $A_{\text{quartz}}$. However, in this study, only a small fraction of the area $A_{\text{quartz}}$ is irradiated by the laser light. Therefore, we identify $A$ in the Sauerbrey
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equation with the cross section area of the laser beam and start from the simplest possible picture to estimate the thickness $x$ of the depleted water layers:

$$xA = -\frac{\Delta m}{\rho}$$

where $\rho$ is simplified as the normal bulk water density 1 g cm$^{-3}$.

With the calculated mass sensitivity constant:

$$C = \frac{\nu \rho Q}{2f^2} = \frac{3340 \text{ m s}^{-1} \cdot 2.651 \text{ g cm}^{-3}}{2 \cdot (5 \cdot 10^6 \text{ s}^{-1})^2} = 17.7 \text{ ng cm}^{-2} \text{ s}$$

and $\Delta f = 22 \text{ Hz}$, we obtain:

$$x = -\frac{\Delta m}{\rho A} = \frac{C}{\rho n} \Delta f = \frac{17.7 \text{ ng cm}^{-2} \text{ s}}{1 \text{ g cm}^{-3} \cdot 7 \cdot 22 \text{s}^{-1}} = 0.55 \text{ nm}$$

From the diameter of one water molecule (0.28 nm), we estimate that the depleted water layers on the hydrophobic polystyrene surface in air corresponding to a 633 nm laser irradiation (beam vertical to the surface) was about two monolayers.

Depending on the wavelength of the laser light, the sensor surface environment (in air or under water) as well as the surface polarity of the quartz crystal, the thickness of the depleted water layers was different, as reflected in the different values of resonance frequency change $\Delta f$. The results are summarized in Figure 3.4.5. In general, 633 nm laser irradiation showed a more pronounced effect in depleting interfacial water layers than 670 nm laser irradiation did. In addition, the influence of the sensor environment on the depleting effect on hydrophobic surfaces was not as apparent as that on hydrophilic surfaces. Moreover, it was observed that the depleted mass on hydrophobic surfaces was larger than that on hydrophilic surfaces in response to laser light irradiation. These results are in accordance with the picture of interfacial water layers on model hydrophobic and hydrophilic surfaces obtained from previous
studies by us and other groups: Interfacial water molecules are tightly packed on the surfaces. On hydrophobic surfaces the interfacial water layers are ordered and less bounded (except hydrogenated diamond) to the surfaces, whereas on hydrophilic surfaces the interfacial water layers are with high viscosity and are more restricted on the surfaces. In other words, the interfacial water layers on hydrophobic surfaces are easier to be influenced and manipulated than those on hydrophilic surfaces, for instance by the laser light irradiation or additional unorganized bulk water molecules. Therefore, the light induced depleting effect on hydrophobic surfaces is more pronounced than that on hydrophilic surfaces, and the effect on hydrophobic surfaces is more sensitive to the surface environment than that on hydrophilic surfaces.

![Graph](image)

**Figure 3.4.5** Synopsis of changes in sensor resonance frequency $\Delta f$ in response to low-level laser light irradiation (633nm, 670nm) perpendicular to the surface plane on polystyrene (hydrophobic) and gold (hydrophilic), in air and under water.

There remains insecurity that the resonance frequency change of the sensor might arise from the local thermal effect induced from the low-level laser light irradiation, even the applied light intensity is very low and the sensor temperature is kept constant by a sensitive thermostat. This possibility has been excluded in a recent paper by a series of experiments which related the temperature to the change in resonance frequency. It is reported that an increase in temperature resulted in a decreased
resonance frequency of the sensor. This result can be understood in the following way: the Young’s modulus and shear modulus of the sensor would be decreased when the temperature is increase. Therefore, the resonance frequency would decease with the increased temperature. On this basis, if the light induced resonance frequency change is indeed from the thermal effect (probable an increased temperature), one would obtain a decreased resonance frequency. This is exactly opposite to the experimental observation. Therefore, the thermal role in the light induced depleting effect could be excluded, at least on this basis.

Here an alternative approach is provided to exclude the thermal role in the effect: irradiation at an angle of 45° to the surface plane (laser position 2 in Figure 3.4.2), whereby the plane of the laser polarization was either parallel or normal to the sensor surface, thus identical energy density of the laser light was distributed on the surface. QCM was to check the resonance frequency change upon different polarized laser light irradiation on hydrophobic polystyrene and hydrophilic gold surfaces both in air and subaquatically. The results are summarized in Figure 3.4.6.

Figure 3.4.6 Changes in sensor resonance frequency $\Delta f$ in response to low-level laser light irradiation (633nm, 670nm) applied at 45° to the surface plane of polystyrene (hydrophobic) and gold (hydrophilic), in air and under water. Smaller frequency values stem from laser polarization parallel to the sensor plane (P), compared to those obtained from laser polarization normal to the sensor plane (N).
It is noted that there was a significant drop in the frequency shift when the laser polarization was parallel to the sensor plane in comparison with the situation when the laser polarization perpendicular to the sensor surface, in all the measured cases. This observation indicates that the light induced depleting effect is probably not from the thermal effect. In addition, recent experimental evidence using a specific experimental setup and a well calibrated infrared camera (Thermal imager: Testo 880-3, LISA Laser products, Katlenburg-Lindau Germany; accuracy after calibration 0.6 °C) (unpublished research data) indicates that a significant temperature increase, as a cause of the laser irradiation, can be ruled out in the reported laser experiments. Therefore, it is concluded that the increase in sensor resonance frequency upon laser light irradiation is not from a thermal effect. In addition, these results expose the intrinsic interaction of the polarized photons with polarized water dipoles. Regarding the relatively low light intensities it is tempting to ascribe the observed frequency changes to a collective interaction of photons with interfacial water molecules, that is, to a polarization of the electron clouds of the water molecules close to the sensor. More details in the interaction mechanism of low-level laser light with interfacial water layers will be revealed in section 3.5.

As will be shown below, we further calculate the thickness of the depleted water layers in response to low-level laser light irradiation with two different wavelengths on two different substrates in air and under water, using the aforementioned Sauerbrey equation. This is summarized in Table 3.4.1. It is found that with the standard oscillator frequency of 35 MHz the increased frequency of QCM is ranging from 2.5 Hz to 22 Hz upon the low-level laser light irradiation, corresponding to the depletion of water molecules from less than one monolayer up to two monolayers.
Table 3.4.1 Summary of the depleted thickness of the interfacial water layers in response to low-level laser light irradiation when the laser beam is perpendicular (Position 1) and with an incidence of 45° (polarization parallel: P or normal: N) to the sensor surface plane.

In summary, one would expect no difference in sensor frequency in response to low-level laser light in general and different laser polarization in particular, for water molecules with randomly distributed dipole moments. These results support the AFAM study (section 3.3) and provide quantitative information additionally. However, the interaction mechanism of the light with interfacial water layers could not be fully understood so far. For example, it would be important to know why 633 nm laser irradiation had a more pronounced effect in depleting interfacial water layers than 670 nm laser irradiation did. Hence, further study as follows in the next section is needed for clarification.
3.5 Probing nanoscopic interfacial water layers by soft XAS & laser light

As it has been shown in the previous sections and references therein, there is a lot of experimental evidence that low-level laser light interacts with interfacial water layers on materials surfaces with different polarities under ambient conditions – a phenomenon which is unknown for bulk water. However, the precise interaction mechanism of the photons with interfacial water molecules is not fully explored.

X-ray absorption spectroscopy (XAS) is an element-selective technique that provides distinctive information about local electronic structure of atoms. In this section, I use soft XAS to study hydrogen bond structure of water molecules on surfaces and near-surface regions. Soft XAS in combination with moderately intense 670 nm laser light was applied, namely soft XAS based dual technique. The experiments were carried out on the hydrogenated nanocrystalline diamond and transparent polystyrene surfaces under ambient conditions. The results support the previous light interaction studies (section 3.3 and section 3.4), and provide “electronic evidence” of the interaction mechanism. It is demonstrated that the molecular structure change in the interfacial water layers is due to the collective hydrogen bond excitation upon laser light irradiation, confirming the initial model as suggested in section 3.4.

3.5.1 Soft X-ray absorption spectroscopy (XAS)

XAS involves the excitation of strongly bound electrons that belong to deep states (core electrons) to weakly bound valence states. According to the core electron which is excited (principal quantum numbers n=1, 2, and 3), K-, L- and M-edges are named respectively. Since the excitation energy depends on the atomic number of the absorbing atom, XAS is element selective and probes local electronic structure of a given atom. In addition, X-ray absorption process is faster than one femtosecond.
XAS spectrum can be regarded as a snapshot of the atomic or molecular orbital structure.

In the soft XAS experiments, soft X-ray beams (photon energy in the range of 100–3000 eV), which are tunable, are usually provided by synchrotron radiation sources. For detection, one can use the effect of relaxation of the excited electrons, that is, electron relaxes and the surplus energy is again emitted as light (fluorescence). Fluorescence yield obtained from soft XAS is bulk sensitive and very suitable for probing thin film structures. It works well for K-edge of carbon, oxygen and fluorine for which the self-absorption effect is small.\textsuperscript{123} In this study, soft XAS is used to probe interfacial water layers by measuring the fluorescence yield X-ray absorption (XA) at the oxygen K-edge of water.

### 3.5.2 Soft XAS on hydrogenated nanocrystalline diamond surfaces

The experiments were performed at the Berlin synchrotron radiation facility (Bessy II) third generation light source beamline U41-PGM, using the recently constructed Liquidrom ambient setup.\textsuperscript{124,125} The advantage of this technique is that the sample of interest can be kept at room temperature and atmospheric pressure. The hydrogenated nanocrystalline diamond used to examine interfacial water layers was with the average grain size of 15 nm and root mean square roughness of 18 nm.

At first, ultrapure water from a nanospray system was used to establish elevated humidity conditions close to the surface of the sample (hydrogenated nanocrystalline diamond or crystal clear polystyrene) in the experimental chamber. The evaporation process was monitored by measuring the XA fluorescence yield at the oxygen K-edge of water. The XA fluorescence signal was detected by a GaAsP photodiode type Hamamatsu G1127 placed at an angle of 45° and a distance of ~ 20 mm from the
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surface of the sample. Figure 3.5.1 shows the photograph of experimental setup. In the interaction chamber, a nanospray of ultrapure water was deposited on the surface of sample, under an atmosphere of neat helium at ambient pressure (~ 930 mbar). Immediately after the spray, the water evaporated from the surface of the sample, until an equilibrium state with the content of gas-phase water was reached, which defined the relative humidity within the chamber. The equilibrium state was controlled by purging the chamber with a continuous flow of fresh helium, which allowed to perform the experiments at arbitrarily chosen humidity conditions.

![Image of experimental setup](image)

**Figure 3.5.1** Photograph of the soft XAS chamber with the nanospray needle for calibration of the controlled humidity conditions. The needle is pointing to the surface of the sample.

Figure 3.5.2 shows a series of XA fluorescence yield spectra in the time window from immediately to 60 min after spray injection of water, showing the evaporation process of the system until equilibrium conditions (red to blue spectra). For comparison the XA fluorescence yield spectrum of liquid water is taken from the literature,\textsuperscript{126} presenting with the strong pre-edge peak at 535 eV, the main-edge peak at 537.5 eV, and the post-edge peak around 541.5 eV (black spectrum). During the process of evaporation, all XA spectra show strong contributions from the oxygen gas (O\textsubscript{2} labeled in Figure 3.5.2) and water vapor (4a\textsubscript{1}, 2b\textsubscript{2}, and 3b\textsubscript{2} molecular gas-phase
orbitals, labeled in Figure 3.5.2). The sources of oxygen gas are traces of oxygen remaining in the chamber after evacuation and purging with helium.

![Figure 3.5.2](image)

**Figure 3.5.2** Series of XA fluorescence yield spectra in the evaporation process after spray deposition of water.$^{127}$

In the next step, the aim is to probe interfacial water layers on hydrogenated nanocrystalline diamond with soft XAS based dual technique. As suggested by the results of AFAM experiments (section 3.3), a sufficient humidity level is a precondition for the observation of the light interaction effect. Therefore, a cotton roll was used as a local moisture buffer, which was brought close to the sample (~ 3 mm) and loaded with water by the spray injection. The slow evaporation rate of the moist cotton at ambient pressure facilitates the adjustment of the relative humidity, together
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with the continuous flowing helium. In order to induce the interaction effect between the light and the interfacial water layers, the sample was irradiated with a 670 nm laser (power 4.5 mW, spot diameter 1.2 mm and intensity 3.98 kW·m⁻²). Additionally, the photodiode detector was shielded against stray light from the laser by means of an aluminum foil with a thickness of 500 nm. The experimental setup is illustrated in Figure 3.5.3a.

Interfacial water layers on the surface of hydrogenated nanocrystalline diamond were investigated at a chosen post-edge photon energy of 541.8 eV (marked by red dashed line in Figure 3.5.2) to avoid interference of the gas-phase water signals. Figure 3.5.3b shows representatively the result of a time scan of XA fluorescence yield signal with and without 670 nm laser irradiation. When the laser light was switched on, an instant increase of XA intensity signal was detected and an equally sudden decrease to previous intensity was monitored when the laser light was switched off (red line in Figure 3.5.3b). In order to avoid artifacts from stray light, a corresponding time scan without X-ray excitation was performed and presented in Figure 3.5.3b in blue line. It shows no correlation with laser light irradiation, thus proving the efficiency of the diode shielding. The effects were tested and reproducible at an energy range of 540 – 545 eV (the post-edge of liquid water, see Figure 3.5.2), also on polystyrene surfaces.
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Figure 3.5.3 (a) Experimental setup of soft XAS based dual technique. (b) A time scan of XA intensity at photon energy of 541.8 eV with and without 670 nm laser irradiation on the hydrogenated nanocrystalline diamond surfaces. (c) Schematic drawing illustrates the interaction of low-level laser light with interfacial water molecules.\(^{127}\)

In general, the induced increase in XA fluoresce yield signal at the oxygen K-edge can be related with an increase in the orbital hybridization of the hydrogen bonding by stretching the hydrogen bond between the water molecules.\(^{128}\) This stretching would correlate with a macroscopic volume expansion. In fact, bulk water shows an extremely weak absorption for visible light, such as 670 nm. This is attributed
primarily to the combinations of harmonics and fundamentals of stretching and bending modes.\textsuperscript{57,58} This has been described in section 3.1.1. However, these effects are too weak to provoke a significant structural phase transition of the molecular network. Therefore, it is concluded that the transition is triggered by a different modality of interaction.

In order to understand the light interaction mechanism, it is important to discuss the aforementioned experimental observations (NSOM, AFAM, QCM and XAS) in a comprehensive manner. First, the precondition of the light interaction effect is the relative humidity. When the relative humidity is very low, the interfacial water molecules seem to be strongly bounded to the surface and thereby not affected by the laser light irradiation, corresponding to a relatively thin water mask. Second, the light interaction effect does not depend on specific substrates, as demonstrated by a variety of surfaces with different polarities. Thus, the activation is not due to a specific molecular ordering of the adsorbed water imprinted by the material surface. Third, the possibility of thermal effect, that is, heating the substrates by the laser light has been obviated in the light interaction effect.

These findings suggest that the light interaction effect may be interpreted in terms of the intermediated structure of the interfacial water molecules, facilitating a collective excitation of the molecular network, confirming the initial model.\textsuperscript{121} This is neither possible in bulk water due to the higher degree of random motion and the fast dissipation of (vibrational) energy nor in the tightly bounded first adsorbed layer on the surface. In short, the light induced effect appears as a breathing-like expansion of the topmost molecular water sheets, as illustrated in Figure 3.5.3c.

This concept receives support from the QCM study (section 3.4). When the polarization is normal to the surface, it resulted in a stronger response. This indicates that there is an enhanced molecular ordering along the same direction. In such terms, the observed structural change of the interfacial water layers may be assigned to an
energy transfer of the O – H stretching mode excitation over many water molecules. This is mediated by dipole-dipole interactions, thus leading to longer collective persistence of excited states of the water molecules. With this mechanism, it could also be able to provide an answer to the question raised at the end of section 3.4, that is, why 633 nm laser irradiation showed a more pronounced effect than 670 nm laser irradiation did. It is probably simply due to the higher photon energy, corresponding to the shorter wavelength. The slightly influence from the surface properties, such as polarity and surface charge can also be understood: the different molecular structure of interfacial water layers oriented by different substrates may respond slightly different in the electrical structure change upon laser light irradiation.

3.6 Conclusions

In this chapter, I presented four different methods to investigate and characterize nanoscopic interfacial water layers on model surfaces with different polarities in air and under water. Each method is with its own advantages and disadvantages. Table 3.6.1 gives a summary of the four methods.

<table>
<thead>
<tr>
<th>Method</th>
<th>Advantages</th>
<th>Disadvantages</th>
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<tbody>
<tr>
<td>Electrical conductivity</td>
<td>Only for hydrogenated diamond</td>
<td></td>
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<tr>
<td>experiments</td>
<td></td>
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</tr>
<tr>
<td>AFAM based dual technique</td>
<td>All smooth surfaces</td>
<td>Presently only in air</td>
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<tr>
<td>QCM based dual technique</td>
<td>• In air &amp; under water</td>
<td>Limited surfaces</td>
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<td></td>
<td>• Quantitative information</td>
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<tr>
<td>Soft XAS based dual technique</td>
<td>Providing the interaction mechanism of</td>
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<td>visible light with interfacial water layers</td>
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Table 3.6.1 Comparison of the four methods in probing nanoscopic interfacial water layers.
After scrutinizing the experimental results in a comparative manner, the following picture is obtained: in general, interfacial water layers prevail on all the solid surfaces in air and under water under ambient conditions. The presence of the interface is more important than the geometry of the interface in influencing the structure of interfacial water layers. Due to their unilateral restriction in mobility, interfacial water molecules are densely packed on the surfaces and presenting molecular structure different from that of the liquid, gaseous and solid forms. Hence, they have unusual physical properties. Depending on the surface polarity of the substrates, their characterizations vary to an extent. In short, on hydrophobic surfaces, they are ordered and less bounded to the surfaces; on hydrophilic surfaces, they are ordered, strongly adsorbed to the surfaces and show high viscosity.

A most striking property of interfacial water layers is their interaction with visible light. This is also the ground that they can be probed by the dual technique, where visible light is used to modulate the molecular structure of interfacial water layers and simultaneously the change is monitored by AFAM, QCM and soft XAS, respectively. Notably, QCM measurements exposed that the thickness of interfacial water layers is in the range of a few nanometers. Soft XAS experiments exploited the light interaction mechanism: visible light irradiation induces a collective hydrogen bond excitation in the interfacial water molecules, in accordance with a breathing-like volume expansion as a pictorial expression. Importantly, the light interaction property can be applied in biological systems, namely, using visible light to modulate molecular structure of subaquatic interfacial water layers in the living cells. This brings an enormous potential for applications in nanomedicine, as will be discussed in chapter 5.

Nanoscopic interfacial water layers on hydrogenated nanocrystalline diamond surfaces are of particular interest and have been comprehensively studied in this chapter. As revealed by the electrical conductivity analysis and AFAM experiments, they are extremely ordered and stable, apparently due to the strong C – H covalent
bond induced by the hydrogen atoms on the surface of hydrogenated diamond. In view of other extraordinary properties of diamond (as described in chapter 2), hydrogenated nanocrystalline diamond is regarded as an amazing biomaterial, which will be discussed in details in the next chapter.
Interface between materials and biological systems: nanoscopic interfacial water layers
Biological applications of nanocrystalline diamond layers

Diamond is one of the allotropic forms of carbon, the principal component of living organisms. Therefore diamond is prepared to be a biomaterial intrinsically. In addition, diamond is a particularly attractive material due to the unique combination of its outstanding properties. Details have been described in Chapter 2. Nanocrystalline diamond layers are the benefits of nanotechnology and open new horizons for the biological applications of diamond. Examples of these applications are biosensors, coatings on biocompatible implants, such as orthopedic implants and artificial heart valves, as well as coatings on medical instruments such as surgical blades.

In this chapter, novel biological applications of nanocrystalline diamond layers are explored by using the results obtained from the interface study (chapter 3). At the beginning (section 4.1), the different definitions of biocompatibility are introduced. A new functional definition of biocompatibility is provided: the biomimetic table, which consists of eight quantitative parameters for a cell’s native physiochemical micro- and nano-environment and is instrumental in designing biomaterials. Nanoscopic interfacial water layers are listed as the first determinant in the biomimetic table. Furthermore, standard biocompatibility and hemocompatibility tests are described in this section using nanocrystalline diamond layers with different chemical terminations (hydrogen, oxygen and fluorine) as well as different surface topography (roughness). Hence, the biosuitability of nanocrystalline diamond layers is evaluated in vitro.

Section 4.2 presents a comparative study of non-hydrogenated and hydrogenated nanocrystalline diamond in the first contact events between the cells and materials. Higher cell survival rates were found on hydrogenated diamond in comparison with those on non-hydrogenated diamond which are with the same surface profile. Considering the highly ordered and stable interfacial water layers on hydrogenated nanocrystalline diamond (one result from chapter 3), this cell culture observation
exposes the decisive role of interfacial water layers in the biomedical applications of biomaterials.

On this basis, two new biological applications of nanocrystalline diamond layers were developed: first, a high-throughput method based on 3D diamond Petri dish (strawberry patterned diamond) was developed to culture embryoid bodies efficiently; second, a new generation of cell culture devices based on diamond was suggested by showing high cell performance on diamond substrates (section 4.4).

4.1 Biological characterization of nanocrystalline diamond layers

4.1.1 Extended definition of biocompatibility: the biomimetic table

Biocompatibility is the key factor in the application of the biomaterials, which are non-viable and/or viable materials used in biomedical devices and intended to interact with biological systems. At present, biocompatibility tests are conducted according to the classical trial and error protocol – an extremely time consuming and expensive process. Traditionally, these tests comprise three successive phases: cell culture experiments, animal tests and clinical studies.

Biocompatibility is a complicated and challenging issue, especially at the nanoscale. For example, nanocrystalline diamond layers are extensively biocompatible both at tissue and cellular level, whereas diamond nanoparticles show excellent biocompatibility at cellular level as verified in a variety of cell lines, but genotoxicity in the embryonic stem cells as showed in a recent study. The complexity of biocompatibility is also reflected in the concurrently used five definitions:
(1) The ability of a material to perform with an appropriate host response in a specific application (Williams’ definition 1999).

(2) The quality of not having toxic or injurious effects on biological systems (Dorland’s Medical Dictionary).

(3) Comparison of the tissue response produced through the close association of the implanted candidate material to its implant site within the host animal to that tissue response recognized and established as suitable with control materials (ASTM International).

(4) Refers to the ability of a biomaterial to perform its desired function with respect to a medical therapy, without eliciting any undesirable local or systemic effects in the recipient or beneficiary of that therapy, but generating the most appropriate beneficial cellular or tissue response in that specific situation, and optimizing the clinically relevant performance of that therapy (Williams DF, Biomaterials, 29, 2941, 2008).

(5) Biocompatibility is the capability of a prosthesis implanted in the body to exist in harmony with tissue without causing deleterious changes (International Dictionary of Medicine and Biology).

Among these definitions, the most prominent one is the Williams’ definition. All these definitions have in common that they are qualitative and describe the result of biocompatibility tests. Practically, biomaterials used for medical devices must be tested according to ISO 109931 (Biological evaluation of medical devices), which is the most widely used standard for assessing the biocompatibility. Therefore, it is possible to avoid unnecessary animal experiments and potential adverse effects (acute and/or chronic) for patients by using these in vitro laboratory tests. In the next sections, in vitro tests for biocompatibility and hemocompatibility are described using different types of nanocrystalline diamond layers.

Here we offer a new approach of the biocompatibility concept using the biomimetic principle: instead of monitoring the responses of cells to their environment (classical
approach), we try to provide a quantitative description of the native environment of cells – for each cell its own and typical environment. We start with analyzing biological cells in their native environment. Biological materials are hierarchically organized composites. They are living components (cells) imbedded in a nonliving extracellular matrix that is built and remodeled by the cells. Notably, the extracellular matrix itself can be also a composite material with organic and inorganic constituents and has the function of providing framework to hold and protect cells. The size of most cells is at the micrometer length scale, but the organelles and macromolecules inside of the cell and on the cell membrane, as well as the components of the extracellular matrix including individual tropocollagen molecules and bone mineral platelets, are several orders of magnitude smaller (at the nanoscale). For anchorage dependent cells, the main parameters of their microenvironment are the composition of the extracellular matrix, chemical signals from soluble factors and neighboring homo- or heterotypic cells, the topography of the substrate. In the biomaterials research, various of cell/biomaterial interaction experiments have shown that the cells behavior are greatly influenced by the surface properties of the biomaterials, such as chemistry, wettability/surface charge and topography (microstructure and nanostructure). In addition, cells respond to mechanical stimulation has been studied in a variety of laboratory experiments. For instance, it has been reported that the mechanical microenvironment (tissue level elasticity) have a strong influence in the naive mesenchymal stem cells lineage specification. Hardness can reflect wear properties of the material. Moreover, the preliminary experiments also showed that hardness may be important for cell behavior. Detailed information is provided later by colleagues in our institute (see chapter 4.4.3). Furthermore, for biodurable materials, the ability to remain unchanged in the corrosive biological environment is the precondition for further research and applications. For instance, bulk metallic glass has been used by us to study biodurability. Currently, bulk metallic glass receives indirect interest in the scientific community from the discovery of
Biological applications of nanocrystalline diamond layers

quasicrystalline structure by Daniel Shechtman, the Nobel Prize winner in Chemistry 2011. Bulk metallic glasses are “designer materials” and some of them are biodegradable. In addition, nanoscopic interfacial water layers, has been only recently accessed experimentally, which is the focus of chapter 3.

Summarizing the individual element, the result of the effort is the biomimetic table (Table 4.1.1). The biomimetic table is a construct of 8 physical and chemical parameters: determinants of biocompatibility. In fact, in the literature other authors also consider biomimetic for the biomaterials research. For instance, it has been reported that by understanding the evolutionary principles and functional properties in biological systems, together with the micro/ultrastructure and theoretical stimulation, a clear strategy for synthesizing optimum artificial attachment structures is made. Importantly, the value of the biomimetic table is not only providing the quantitative testing parameters but also a powerful strategy for designing biomaterials, as will be shown in section 4.3.

| 1. Nanoscopic interfacial water layer | important in the first contact events (see section 4.2) |
| 2. Nanostructure | cells are sensitive to nanostructure of the substrate$^{136,138,139}$ |
| 3. Surface charge | related to interfacial pH, hydrophobicity/hydrophilicity$^{135,145}$ |
| 4. Chemistry | cells are sensitive to chemical signals$^{139}$ |
| 5. Microstructure | cells are sensitive to microstructure of the substrate$^{135,137}$ |
| 6. Elasticity | cells respond to change of Young’s Modulus$^{139,141}$ |
| 7. Hardness | reflecting wear properties$^{142}$ |
| 8. Biodurability | durability in biological environment |

Table 4.1.1 The biomimetic table consists of 8 determinants of biocompatibility.
4.1.2 Biocompatibility on nanocrystalline diamond layers

On the basis of the 8 determinants listed in the biomimetic table, nanocrystalline diamond layers can be supposed in general biocompatible at cellular levels. In this section, L929 mouse fibroblast cells (an adherent cell line) were used to evaluate biocompatibility of six different nanocrystalline diamond layers: nanocrystalline diamond (DR), hydrogen (HR), oxygen (OR) and fluorine (FR) terminated nanocrystalline diamond with the average grain size of 250 nm and root mean square roughness of 37 nm; nanocrystalline diamond (DS) and hydrogen (HS) terminated nanocrystalline diamond with the average grain size of 15 nm and root mean square roughness of 18 nm.

Two different established in vitro toxicity tests (agarose overlay assay and XTT assay), followed the instruction given in DIN EN ISO 10993-5:2009 (Biologische Beurteilung von Medizinprodukten – Teil 5: Prüfungen auf In-vitro-Zytotoxizität), as well as a period of 7 days cell adhesion and proliferation experiments were applied to elucidate the biocompatibility of the nanocrystalline diamond layers.

4.1.2.1 Agarose overlay assay

Agarose overlay assay is a standard procedure to test the cytotoxicity of biomaterials qualitatively. A neutral red solution is brought into the metabolic active cells and works as a signal.

Here the experimental procedure was as follows. $5 \times 10^5$ L929 cells were seeded on Petri dishes (60 mm) and cultivated for 48 h without cell culture medium exchange. An incomplete monolayer was formed and then covered by 4 ml 1.5 % nutrient layer agarose. The thickness of the agarose layer was 0.14 cm based on the calculation according to the formula: volume = $\pi \times \text{radius}^2 \times \text{height}$. After the agarose layer was solidified (30 min) at room temperature, 2 ml of freshly prepared 0.01 % neutral red
solution in sterile phosphate buffered saline (PBS) was added to the Petri dishes and incubated for 30 min at 37 °C. The red solution was then removed by aspiration via a pipette. Cell staining and cytomorphology were microscopically assessed. The tested diamond samples were sterilized in 70% ethanol and then placed on the top of the agarose gel and incubated for 24 h under standard cell culture conditions (37 °C, 5% CO₂ and saturated humidity). The cells were examined by light microscopy. A sterile cover glass (nontoxic) was used as negative control and a toxic bulk metallic glass was used as positive control.

The light microscopy images showed that for all the tested nanocrystalline diamond layers and negative control, almost all cells (> 95%) were vital (red colored), showing a typical spindle shaped of L929 cells (see Figure 4.1.1), whereas for the positive control, there were no cells in red. This means that all the tested nanocrystalline diamond samples showed no cytotoxicity.

Figure 4.1.1 Morphology of L929 cells after incubation with the nanocrystalline diamond sample (DS). Abbreviation is described at the beginning of section 4.1.2.
4.1.2.2 XTT assay

The principle of XTT assay is that viable cells will take up tetrazolium salt XTT: 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilid, through the active dehydrogenases of the mitochondria, thereby converting XTT into an orange colored water soluble formazan product, which can be measured and quantitated by spectrophotometer.

Here the experimental procedure was as follows. $2 \times 10^4$ L929 cells per well were seeded into 96-well plates and then incubated for 24 h under standard cell culture conditions. The tested nanocrystalline diamond samples were placed into 24-well plates with 1 ml cell culture medium per well and located in the incubator for 24 h. After 24 h, the aliquots were harvested from the medium contacting the test samples and transferred into the 96-well plates. Cells were then cultured for 24 h under standard cell culture conditions prior to cytotoxic evaluation. XTT reagent solution was added to each well and the 96-well plates were incubated in the incubator for another 2 hours. A spectrophotometer SpectraMax M2 (Molecular Devices) was used to measure the absorbance of the samples at 440 nm. The results showed that all the tested nanocrystalline diamond layers were nontoxic (see Figure 4.1.2).

![Graph showing XTT test results for different nanocrystalline diamond layers.](image)

**Figure 4.1.2** XTT test on different nanocrystalline diamond layers. Polystyrene was used as negative control (Medium). Abbreviations are described at the beginning of section 4.1.2.
4.1.2.3 Cell adhesion and proliferation

It is well-known that anchorage-dependent cells, such as L929, do not survive or proliferate without a substrate, and cell adhesion is the precondition for cell activities, such as spreading and proliferation. Here the study of cell growth on the aforementioned six different nanocrystalline diamond layers were performed using L929 cells. The samples were sterilized using 70% ethanol. Cells were seeded at a concentration of $2.5 \times 10^4$ cells/ml and incubated over a period of 7 days under standard cell culture conditions. Polystyrene Petri dishes were used as positive control. Calcein acetoxymethyl ester (calcein AM) was used as fluorescent dye to trace viable cells. A fluorescence microscope (Apotome) was applied to observe cells morphology and proliferation. Figure 4.1.3 displays a fluorescence image taken on nanocrystalline diamond surface (DS). In sum, the cells showed good cell adhesion and proliferation on all the tested nanocrystalline diamond layers.

Figure 4.1.3 Fluorescence microscopy image of L929 cells on the nanocrystalline diamond film (DS) after 7 days culture. Abbreviation is described at the beginning of section 4.1.2.
4.1.3 Hemocompatibility on nanocrystalline diamond layers

Hemocompatibility is a biocompatibility of a material when it is in contact with blood. The ideal hemocompatible material should present no or very weak interaction between the material and blood, thereby preventing blood clotting or thrombus formation and keep the blood flow in the intervened area. Of note, the events that determine hemocompatibility often occur at the molecular level. It is well-known that the interaction of proteins and surface is a fundamental phenomenon that determines the subsequent cell response, and it changes with surface properties, such as topography (roughness) and surface polarity (hydrophilic or hydrophobic).\textsuperscript{145,150,151} Here albumin and fibrinogen adsorption test as well as thrombocytes adhesion experiments were performed to investigate the hemocompatibility of the above-mentioned six different nanocrystalline diamond layers.

4.1.3.1 Albumin and fibrinogen adsorption test

Albumin is a water soluble protein in the blood plasma. Its adhesion on the substrates can prevent the adhesion of thrombocytes. As a contrary, fibrinogen is a water soluble protein that can enhance the adhesion and activation of thrombocytes. Thrombus can be induced when fibrinogen is converted into insoluble fibrin polymer by the action of the enzyme thrombin. In other words, strong albumin adsorption relates to low thrombogenicity (good hemocompatibility); strong fibrinogen adsorption indicates high thrombogenicity (low hemocompatibility).

The albumin and fibrinogen adsorption on nanocrystalline diamond surfaces with different terminations and surface profiles was examined by inverted enzyme-linked immunosorbert assay (ELILA).\textsuperscript{44} In the experiments, all the tested diamond samples and the silicon wafers were incubated in three major steps, including standardized blood plasma, primary antibodies (anti-human albumin or anti-human fibrinogen), and
peroxidase-linked secondary antibodies. After rinsing off the unbound antibody enzyme conjugates, a stain was added to convert the enzyme into a color signal, followed by sodium dodecyl sulfate (SDS) to stop this conversion. The amount of adsorption proteins was proportional to the remaining stain, which was quantified by measuring the absorption of the solutions at 405 nm using a spectrophotometer SpectraMax M2 (Molecular Devices). The absorption values obtained from different diamond samples were normalized relative to silicon substrates.

Figure 4.1.4 and 4.1.5 show that the adsorption of albumin and fibrinogen on the tested nanocrystalline diamond substrates is in general stronger than that on silicon substrates and indicate moderate to strong thrombogenicity of the nanocrystalline diamond substrates after analyzing the adsorption ratio of albumin to fibrinogen. The oxygen terminated nanocrystalline diamond (OR) showed higher albumin/fibrinogen ration (improved hemocompatibility), which may attribute to the hydrophilic nature of the surface.44

![Figure 4.1.4 Relative adsorption of albumin on different nanocrystalline diamond layers. Abbreviations are described at the beginning of section 4.1.2.](image)
4.1.3.2 Thrombocytes adhesion test

When blood comes into contact with a foreign material, thrombocytes may adhere to the surface depending on the constitution of the previously adsorbed plasma protein layer. The evaluation of the thrombocytes adhesion on the surface of a biomaterial is an important access to the hemocompatibility of this biomaterial. A low thrombocytes adhesion refers to good hemocompatibility while a high thrombocytes adhesion may result in the formation of thrombus (low hemocompatibility).

In this test, nanocrystalline diamond samples were incubated using platelet rich plasma from fresh blood contributed by healthy volunteers. The thrombocytes counts were determined by a flow cytometry to be $5 \times 10^4$ cells/$\mu$l. The cells were fixated with glutaraldehyde (to induce autofluorescence)\textsuperscript{152} for 30 min at room temperature, washed twice with PBS and subsequently imaged and counted using fluorescence microscopy (Apotome, image J).
Figure 4.1.6 Fluorescence microscopy image of thrombocytes on silicon wafer.

Figure 4.1.6 presents the morphology of the adhered thrombocytes on silicon wafers. According to the categorization in morphology for the adherent thrombocytes,\textsuperscript{152} most of the cells here belong to catalog IV (spreading or late pseudopodial) or V (fully spread). Figure 4.1.7 shows that thrombocytes adhesion on nanocrystalline diamond and silicon substrates was in the range from moderate to strong, consistent with the results of albumin and fibrinogen adsorption test. Correspondingly, most of the diamond samples showed a low hemocompatibility, with an exception of oxygen terminated nanocrystalline diamond (OR). The result may suggest that oxygenated nanocrystalline diamond coatings may be suitable for endovascular devices. However, this thrombocytes adhesion test was performed under static conditions, where the thrombogenic properties are different from the in vivo situation.\textsuperscript{153} Therefore further research including experiments under dynamic conditions is needed.
4.1.4 Conclusions

In the cell culture experiments, the biocompatibility of nanocrystalline diamond layers with different chemical terminations as well as different surface roughnesses was examined. Two different cytotoxicity tests were performed using L929 cells: agarose overlay assay (qualitatively) and XTT assay (quantitatively). In addition, hemocompatibility tests with human thrombocytes were performed. Protein competitive adsorption tests using albumin and fibrinogen were also made. The results of these tests demonstrate that nanocrystalline diamond layers have good biocompatibility and relatively poor hemocompatibility. Consequently, the basic biological properties of nanocrystalline diamond layers are characterized, which lays the groundwork for further investigations on the biological applications of nanocrystalline diamond layers.

Figure 4.1.7 The number of adherent thrombocytes on different nanocrystalline diamond layers and silicon substrates. Abbreviations are described at the beginning of section 4.1.2.
4.2 First contact events between cells and nanocrystalline diamond layers

A core major challenge in biocompatibility research is biointegration between implant and tissue.\textsuperscript{154} The root cause of biointegration can be attributed to the first contact events which take place at the first moment when the implant is placed into the biological system. Within milliseconds, a biolayer consisting of water, hydrated proteins and other biomolecules from physiological fluids is formed on the surface of the biomaterial, which will initiate the further interaction between cells (tissue) and implant. The characterization of the biolayer is determined by the physical, chemical and biological properties of the implant surface.\textsuperscript{155}

In order to achieve better biointegration and biocompatibility, considerable efforts have been made to understand and modulate this biolayer. The focus of most research is by providing specific bioactive coating on the surface of the implant to enhance the adhesion of wanted cells, and possibly prevent attachment of other cells.\textsuperscript{156-158}

The role of interfacial water layers on this topic has never been studied systematically, although its importance has been addressed by many individual studies which describe that in physiological milieu, water is not an inert environment but rather an integral component of proteins and actively involved in protein actions\textsuperscript{21,159-161} – one key factor in the first contact events between cells and biomaterials. A very recent paper reports on a master-slave concept in which it is believed that interfacial water layers control the properties and thereby behaviors of proteins.\textsuperscript{24} In fact, the first contact between cells and biomaterials would be the contact between their masking water layers (as illustrated in Figure 4.2.1), which are regarded as blueprint of the underlying surfaces\textsuperscript{22} and have an important role in the biocompatibility.\textsuperscript{23}
Biological applications of nanocrystalline diamond layers

**Figure 4.2.1** The role of interfacial water layers during first contact events between the cells and biomaterials.

Here rat neuronal cells PC12 are used as model cells to study the first contact events between the cells and nanocrystalline diamond layers. PC12 cells were seeded onto hydrogenated diamond and non-hydrogenated diamond with identical surface profile (average grain size of 250 nm and root mean square roughness of 37 nm), respectively. Figure 4.2.2 shows the cell morphology directly after seeding. It was observed that massive cell debris (arrow in Figure 4.2.2a) on diamond surface and on the contrary very high cell survival rate on hydrogenated diamond surface.

We propose in the following a simple scenario that provides a consistent explanation for the remarkable results. As suggested by electrical conductivity analysis (section 3.2) and AFAM experiments (section 3.3.3), the nanoscopic interfacial water layers on hydrogenated diamond surfaces are more ordered and stable than those on diamond surfaces due to the polarizing bond to the hydrogen atoms. For living cells seeded on hydrogenated diamond surfaces, such interfacial water layers should act as protective viscoelastic blankets, transiently shielding the hydrogenated site from direct contact with cells. Considering the well documented characterization of PC12 cells that they are loosely bounded in the cell culture milieu and generally requiring
special extracellular matrix proteins (such as collagen) coated on the plastic tissue culture dishes to enhance cell adhesion,\textsuperscript{162,163} the observation that during first contact higher survival rates found on the hydrogenated diamond surfaces indicates that the masking interfacial water layers work as damping elements and are with superior stability against breaking compared to those on diamond surfaces. During the first contact events between the cells and materials, interfacial water layers are probably instrumental in facilitating metabolic processes at the cell’s basal side by softening the contact to the solid diamond, thereby enhancing the cell’s survival rate. In addition, in long term culture conditions PC12 cells presented a surprisingly high affinity for nanocrystalline diamond, which is supposed to be induced by the hydrogen atoms on the substrates (even without additional hydrogen termination), providing the active chemical bonds for molecules on the surface of the cells.\textsuperscript{164}

![Figure 4.2.2](image)

**Figure 4.2.2** Light microscopy photographs of PC12 cells directly after seeding on (a) nanocrystalline diamond, and (b) hydrogenated nanocrystalline diamond layers. Red arrow shows cell debris.\textsuperscript{80}

In summary, the cell culture observation supports the previous laboratory experimental results (chapter 3) and evidences that interfacial water layers are important mediators during the first contact events where cells decide between survival and apoptosis. Importantly, the higher the product density, thickness, stability of the interfacial water layers on a biocompatible material, the higher the expected
cell survival rate, depending on the specific cell-biomaterial system. Hydrogenated nanocrystalline diamond substrate is particularly interesting from the combination of the hydrophobicity at the macroscopic scale and the ability of binding and polarizing water molecules at the nanoscale. The results suggest that moderate hydrogenation of conventional implants may improve biointegration.

4.3 Embryoid bodies built on strawberry patterned nanocrystalline diamond

Stem cell research receives much attention because of the broad potential benefits in the scientific understanding of the cellular basis of human development as well as the therapeutic applications. It promises to provide a novel approach to understand pathogenesis of disease or treatment of varieties of diseases by replenishing tissue, which opens a new era of regenerative medicine.\textsuperscript{165-168}

Routinely, stem cell research is done in Petri dishes, mainly made of polystyrene. With the development of new biomaterials, a multitude of biocompatible materials has also been applied in stem cell experiments. However, these are generally two-dimensional, unnatural milieu for cells and thereby fundamentally limiting the function of the substrates to mimic the complicated in vivo situations. Of note, one of the most prominent methods to study stem cell differentiation is to let embryonic stem cells to form embryoid bodies, which are three-dimensional multicellular aggregates of differentiated and/or undifferentiated cells, similar to an early embryonic stage (i.e. morula).\textsuperscript{169-172} However, the mechanism of the formation of embryoid bodies is not completely understood, and to efficiently produce them in the laboratory is in no way trivial – an important technical obstacle in stem cell research. The hanging drop method is the current widely used method of culturing embryoid bodies, which consists of multiple steps. Therefore, researchers are driven to develop new and more
advanced techniques and methods. In this section, I present a simple and reusable system to cultivate embryoid bodies in extremely short times. The method is based on strawberry patterned hydrogenated nanocrystalline diamond layers.

4.3.1 Introduction to stem cells

Stem cells are unspecialized or undifferentiated cells that can self-renew to generate more stem cells or differentiate into diverse specialized cell types. In general, there are two types of stem cells: one is from embryos (embryonic stem cells), and the other type is from adult tissues (adult stem cells). Embryonic stem cells are pluripotent cells derived from the inner cell mass of embryos at the blastocyst stage. They have two fundamental properties: self-renewal (the unlimited ability to divide and generate new stem cells without going into senescence) and to differentiate into nearly all the specialized cell types under appropriate physiological conditions.\(^{173-175}\) In contrast, adult stem cells are mostly multipotent cells, which have limited self-renewal ability and can only differentiate into a limited number of closely related cell types.

Stem cells realize the function of self-renewal by symmetric divisions and proliferative differentiation by asymmetric divisions: a stem cell divides into one father cell that remains a stem cell, ready to produce more stem cells when needed and one daughter cell that differentiates into a specialized type of cell. Asymmetrical cell divisions thereby maintain and/or expand the local stem cell pool while contributing mature differentiated cells for tissue growth or repair.\(^{176}\)

Importantly, preserving the stem cells phenotype depends critically on their regulatory microenvironment or niche. Stem cell niche determines the fate of stem cells, consisting of several components: basement membrane, cell-cell interactions, extracellular matrix, growth factors, cytokines and other regulatory molecules. The interplay of these factors causes stem cells to make the decision between self-renewal and differentiation, as illustrated in Figure 4.3.1. Frequently, the stem cell niche is a
hydrophobic or moderately hydrophobic and “quiet” (in terms of no chemical changes) environment. For example, follicular stem cells reside continuously in a virtually hydrophobic milieu, proximal to the sebaceous gland producing and containing the hydrophobic sebum, thereby protecting from reactive oxygen species.\textsuperscript{177-180}

Figure 4.3.1 Stem cell niche signal controls the fate of a stem cell: self-renewal or differentiation.

In sum, stem cells serve as the fundamental cornerstone during the life developmental processes and the body’s silent reserves by the continuous supply of new cells to replace the damaged cells whereby maintaining the tissue homeostasis. Stem cell research potentially offers treatment of many diseases, such as Parkinson’s disease, cardiac failure, and diabetes, and provides new strategies for regenerative medicine.

In stem cell research, mouse embryonal carcinoma cells P19 are often used as a model system to study the cells both in differentiated and undifferentiated states for three reasons. First, P19 cells are pluripotent stem cells with well established characterization. They can be efficiently induced to differentiate into various cell types of endodermal, ectodermal and mesodermal germ layers by simple manipulation of culture conditions. For example, the cells can be induced to differentiate into neural cells in the presence of retinoid acid and cardiac cells with dimethyl sulfoxide (DMSO). Second, they can be maintained in serum-supplemented cell culture medium without additional feeder cells to keep them in an undifferentiated state, thereby
allowing the stem cell in vitro research to be carried out at relatively low costs. Third, embryoid bodies can be generated by P19 cells. These are three-dimensional cell aggregates and similar to post-implantation egg-cylinder stage embryos, providing a suitable system for investigating embryonic development at cellular levels and analyzing molecular mechanisms that regulate differentiation.170,172,181-185

4.3.2 Current methods for embryoid bodies formation

Embryoid bodies are in most cases generated in the laboratory by the hanging drop method. The standard experimental procedure is as follows. Drops of cell culture suspension with a defined number of cells are pipetted onto the inverted cover of polystyrene bacterial Petri dish which is moderately hydrophobic, thereby the interaction between the cell and the substrate is low. Cells would aggregate at the bottom of the drops because of gravity. In other words, cells self-assembly would not be hindered by the liquid/air interface. The cover is placed on the Petri dish, which contains PBS, preventing the evaporation of the drops, and cultured in the incubator (37 °C, 5% CO₂ and saturated humidity). After two days incubation, embryoid bodies are formed at the bottom of the drops. The embryoid bodies are then harvested, gently centrifuged, resuspended and incubated in gelatin pretreated culture dishes containing cell culture medium to complete further stages of development. Figure 4.3.2 illustrates the procedure of the standard hanging drop method.

Notably, this method has several disadvantages which have been pointed out during the practical operation. First, the size of an embryoid body is limited due to the constraint of the drop size (the stability of the drop depends on contact angle, drop volume and gravity) and the restriction of the cell concentration (practically there is no possibility to exchange cell culture medium during the formation of the embryoid body). Second, this method consists of several critical steps, which would bring cells
into stressful situations unavoidably.\textsuperscript{186,187} Therefore, new techniques with improved cultivation methods are urgently needed.

**Figure 4.3.2** Schematic drawing of the principle of the hanging drop method.

In the literature, several other alternative methods for producing embryoid bodies have been suggested. Since the key for embryoid body formation is that cells self-assembly should not be hindered, the formation conditions can be in principle created by chemical modification of the materials surfaces and/or geometrical restriction of the biological cells. For instance, highly hydrophobic polymer surfaces, which show low cell adhesive properties are taken into consideration.\textsuperscript{186,187} One of the major disadvantage of this method is that the formed embryoid bodies are not homogeneous. Therefore, it is not in general used. Furthermore, 3D cavities with diameter ranging from millimeter, such as 96-well plates, to micrometer, which are developed through the merging of microscale engineering approaches and biomaterials, has been used to induce the formation of embryoid bodies with controlled size.\textsuperscript{187-190} These 3D cavities
are mainly based on biocompatible polymer with or without additional surface functionalization to inhibit cell attachment. In the next sections, 3D microcavities based on moderately hydrogenated nanocrystalline diamond are described to produce embryoid bodies.

4.3.3 Biomimetic thinking

Biomimetic, bionic or bioinspired thinking refers to mimicking nature or biology, which has been developed since billions of years with a selective optimization process. We can acquire precious knowledge by recognizing the optimized end product and its functionality at the macro, micro and nanoscale. A diversity of materials and structures is created from biomimetic thinking according to a limited number of elements. For example, the nanostructure of gecko setae, which allows gecko adhering well to strongly hydrophobic and hydrophilic surfaces,\textsuperscript{191} inspires the production of dry adhesives, for instance carbon nanotube-based self-cleaning adhesives\textsuperscript{192} and bandages based on biodegradable and biocompatible elastomers coated with oxidized-Dextran.\textsuperscript{193} Superhydrophobic surfaces with extraordinary functions, for instance, self-cleaning are produced by exploiting the secret of lotus leaves’ water-repellency: the interplay of nanostructure (nanohairs), microstructure (microbumps) and chemistry (waxy coating).\textsuperscript{194,195} In addition, subaquatic adhesion and subaquatic superhydrophobicity was observed by immersing a house fly into water. The adhesive setae, which permit the fly to walk under water, are probably hydrophilic due to the protein-secreting microorganisms entrapped in the intersetal space, and are therefore terminated with high viscose nanoscopic interfacial water layers (as learned from chapter 3). The spontaneous formed integral air bag separating the body of the fly from the surrounding water can be traced back to the functional interplay of the same parameters for superhydrophobic surfaces. It is worth mentioning that a small amount of alcohol was added to the water to destroy the air
bag, simultaneously the fly died. The air bubble eliminating function of alcohol was practically exploited in the method of wet medium transfer, which is described in section 4.3.4.2. From the study of the subaquatic fly locomotion, the following potential applications are suggested: advanced contact lenses, non-contact bandages, which could be important for skin burns and miniature submarines.\textsuperscript{196}

As mentioned in section 4.1.1, the biomimetic table is instrumental in designing biomaterials. In an attempt to implement the complete set of 8 parameters into a biologically relevant surface the strawberry model was chosen. In nature, strawberry seeds maturate solitarily in semiprotective superficial niches; thereby they receive nutrients from one side and are exposed to the sun from the other side. This is the principle for a three-dimensional niche, mimicking the space provided by the extracellular matrix to a solitary cell (mechanical support and physical stimuli). In this way, it is plausible to suppose that a number of cells would occupy a preselected microcavity (microstructure).

The combination of biodurability, constant hardness, adjustable surface charge and nanostructure brings nanocrystalline diamond substrates into the focus of current research in life sciences worldwide, in this case, an ideal cell culture substrate.

From the present standard method used to produce embryoid bodies, i.e., hanging drop method, it is learned that embryoid body formation is facilitated by the interplay of good cell-cell adhesion and minimum adhesion between cells and their contact environment, i.e., the liquid-air interface. Here a drop evaporation model (Figure 4.3.3) is used to exploit the bioadhesivity. The evaporation pattern (dot or ring) is the result of the competitive binding between the particle-convective flow interaction and the particle-substrate interaction.

In order to explore the evaporation pattern, drop evaporation experiments were done under ambient conditions. Two equally sized water drops containing particles with different polarities, hydroxyapatite or polystyrene, were applied on the same
hydrophilic titanium substrate. The evaporation results are presented in Figure 4.3.4. Hydroxyapatite particles showed preference to stick to the substrate upon contact whereas polystyrene particles tend to form perfect ring, demonstrating their difference in bioadhesivity.

![Figure 4.3.3](image)

**Figure 4.3.3** Self-explanatory illustration of the parameters controlling the organization of nanospheres in an evaporating sessile water drop: (1) radial convection, (2) nanosphere-substrate interaction, (3) nanosphere-nanosphere interaction, (4) subaquatic water layer, (5) marginal outflow, (6) temperature gradient (∆T), (7) gravity (Fg), (8) substrate, (9) nanospheres, and (10) evaporation time.\(^{197}\)

![Figure 4.3.4](image)

**Figure 4.3.4** (a) Hydroxyapatite microparticles contained water drop evaporation formed dot. (b) Under the same conditions, polystyrene nanospheres formed perfect ring.\(^{198}\)
In consideration of this important aspect (bioadhesivity), the strawberry patterned nanocrystalline diamond samples are moderately hydrogenated due to indications that hydrogenation is instrumental in reducing the first contact attachment between the cells and nanocrystalline diamond substrates, obtained from three independent experiments. First, previous cell culture experiments performed with PC12 cells, showed that cells seeded on hydrogenated nanocrystalline diamond substrates survive better than those on non-hydrogenated nanocrystalline diamond in the first contact events (see section 4.2). Second, the interfacial water molecules prevailed on the surface of hydrogenated nanocrystalline diamond are highly ordered, as indicated by the electrical conductivity experiments (see section 3.2). Third, AFAM experiments performed under ambient conditions provided evidence for the existence and the exceptional stability of the ordered water layers on hydrogenated nanocrystalline diamond, as opposed to those on non-hydrogenated sample of the same surface profile (see section 3.3.3). Based on these considerations we further formulated the paper that hydrogenated diamonds could have played a role as initiators of origin of life on primitive Earth, a work which attracted much attention.

In this context, strawberry patterned moderately hydrogenated nanocrystalline diamond substrates are suggested for the generation of embryoid bodies, as a preferred alternative to the hanging drop method and the current existing methods.
4.3.4 The strawberry patterned diamond method

4.3.4.1 Preparation and characterization of strawberry patterned nanocrystalline diamond layers

The geometry of the strawberry patterned substrates was defined by photolithography. Silicon wafers were structured by deep reactive ion etching. The etched wafers were coated with nanocrystalline diamond, using CVD process with a tungsten hot filament. Details about the fabrication of CVD diamond layers are described in Chapter 2. Hydrogen termination was realized by a hot plasma process. In short, the formation of strawberry patterned nanocrystalline diamond substrates comprises two processes: first, cavity formation on silicon substrate, as illustrated in Figure 4.3.5a-c; second, hydrogenated nanocrystalline diamond layers growing on the silicon substrate, see Figure 4.3.5d. The diameters of the hemispherical cavities were 100 and 200 μm with depth of 50 μm and 100 μm, respectively.

![Figure 4.3.5 Schematic diagram of the experimental procedure of the strawberry patterned nanocrystalline diamond film on silicon substrate.](image_url)
SEM micrographs show the structure and morphology of the strawberry patterned diamond substrates. Figure 4.3.6 illustrates the sample with the cavity diameter of 100 µm, presenting the homogeneous nanocrystalline diamond layers at the bottom of the cavities.

**Figure 4.3.6** SEM images showing the structure of the strawberry patterned diamond substrates and the micrograph of the homogeneous diamond layers at the bottom of the cavities.
4.3.4.2 Embryoid bodies on strawberry patterned diamond

The P19 cells were propagated using the following procedures. Cells were cultured in standard medium, consisting of Dulbecco’s modified Eagle Medium (DMEM), supplemented with 15% fetal calf serum (FCS), 1% β-Mercaptoethanol, 1% glutamine, 1% PS (5000 IU/ml penicillin – 5000µg/ml streptomycin) and 1% non-essential amino acids under standard cell culture conditions. The cells were maintained at subconfluency by subculturing them every 2 days onto tissue culture dishes (hydrophilic polystyrene). 0.2 Trypsin/0.02 EDTA were used to detach cells from the culture dishes.

Embryoid bodies formed from P19 cells were grown according to the hanging drop method (Figure 4.3.7a), harvested after 2 days incubation from hanging drop arrays (Figure 4.3.7b) and subsequently transferred onto the strawberry patterned diamond samples. In this study, 100 µm cavities were too small for the production and culture of embryoid bodies, therefore 200 µm cavities were used. In order to sterilize and prevent the formation of air bubbles in the 200 µm cavities in the diamond samples, the method of wet medium transfer was used before pipetting the medium containing the embryoid bodies onto the diamond samples. The ethanol covering the diamond sample that was placed in a Petri dish was stepwise replaced by ultrapure water in a decreased concentration of ethanol series (70%, 50%, 30%), and finally water.
**Figure 4.3.7** (a) 20 µl drops of cell suspension (containing ca. 125 cells per drop) were placed via pipette on the cover of the bacterial Petri dish. (b) Embryoid bodies were washed down and collected.

The representative light microscopy image in Figure 4.3.8 merges the characteristic strawberry niche pattern with the analogous structure on the diamond holding embryoid bodies grown in the 200 µm cavities after 24 h cell culture incubation. It displays that the embryoid bodies prefer to take place in the cavities.

**Figure 4.3.8** (a) Strawberry biomimetic model. (b) Light microscopy image of strawberry structure in diamond without and with P19 based embryoid bodies seeded onto the substrate.²⁰⁰
In a next step, diamond substrates were seeded with P19 cells at a concentration of \(1 \times 10^6\) cells/ml, determined by a CASY cell counter (Innovatis, Reutlingen, Germany). The diamond substrates were prepared using the previously mentioned wet medium transfer method to sterilize and prevent air bubble formation in the 200 µm cavities. Figure 4.3.9 shows the light microscopy images taken at two different focal planes on the diamond substrate after 2 h incubation of the cells. It is obvious that the number of cells per surface area is smaller in the space between cavities than in the cavities. This may be an indication of the coordinated cell behavior induced by the niche microenvironment.

![Figure 4.3.9](image)

**Figure 4.3.9** Light microscopy images of two different focal planes: (a) the bottom; (b) the surface of the diamond substrate.

After 2 days incubation, the direct formation of embryoid bodies were observed on the diamond substrates by optical microscopy and confocal laser scanning microscopy. Calcein AM was used as fluorescent dye to record the vitality status of cells in embryoid bodies, as it is routinely used to determine the vitality status of voluminous multicellular spheroids. Representative photographs of a cavity-formed embryoid body are shown in Figure 4.3.10.
In this study, it is demonstrated that P19 cells, instead of attaching themselves to the cavity surface, preferred to form aggregates, exactly as in the case of the hanging drop method standardly employed in the embryoid body formation, in which cells have no chance to stick to the liquid-air interface and therefore come together to form aggregates at the bottom of the drop. As shown in this section, the strawberry patterned diamond method is simple and straightforward. However, it is worth mentioning that the seeding concentration of the cells and the size of the cavity are critical for the formation of embryoid bodies or monolayer of cells. For example, a seeding concentration of the cells as low as $1 \times 10^5$ cells/ml was used for strawberry patterned diamond with the cavity diameter of 200 µm. After 5 days incubation, the cells were washed once with PBS, fixed with paraformaldehyde (PFA) for 20 min at room temperature and subsequently washed twice with PBS. The nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) for 5 min at room temperature in the dark and subsequently washed twice in PBS and once in ultrapure water. Confocal fluorescence microscopy images revealed that a confluent layer of cells was realized at the bottom of the cavities (Figure 4.3.11).
Compared to the current methods that are used to form embryoid bodies, that is, the standard hanging drop method and others (see section 4.3.2), the strawberry patterned moderately hydrogenated nanocrystalline diamond is complete materialization of the 8 determinants listed in the biomimetic table and have significant advantages. The hydrogenated diamond substrates carry a highly ordered and stable interfacial water layers (see chapter 3), which would prevent direct contact between the material and the biological cells, therefore showing an improved biocompatibility from the first contact moments (see section 4.2). They facilitate the formation of embryoid bodies with simple operation, thereby avoiding stressful situation for cells. In addition, diamond substrates are robust and can be reusable for indefinite times with appropriate cleaning methods. Moreover, the size of the embryoid bodies can be in principle controlled by the initial cell concentration and the incubation time. Noting that generation of embryoid bodies is the most robust method to provide most differentiated cell types, this study recommends the strawberry patterned diamond substrates as a breeding platform for stem cells – in combination with specific differentiation induce factor, or possibly even without.

**Figure 4.3.11** Confocal fluorescence microscopy image reveals when the initial cell concentration was too low, P19 cells formed a monolayer at the bottom of the cavities.
4.4 Cell culture devices based on nanocrystalline diamond layers

In the previous section, embryoid bodies generated on 3D diamond Petri dish (strawberry patterned diamond) are described. It is worth mentioning one well documented development of the embryoid bodies: with appropriate stimuli and culture conditions, parts of the embryoid bodies would start to differentiate into cardiomyocyte like cells beating in a coordinated way in order to transport nutrients into the core of the increasingly larger embryoid bodies.\textsuperscript{201} In addition, it is reported independently that there is a significant increased cardiomyocyte differentiation from human embryonic stem cells when cultured in the reduced nutrients conditions.\textsuperscript{202} This is interpreted as the social ability of the stem cell system in which a group of individual cells takes a route that secures the survival of the community. Similar social behavior of cells has been found in the case of low oxygen conditions, the embryoid bodies formed from human embryonic stem cells would differentiate into a high number of hematopoietic progenitor cells, the building blocks of the vascular system,\textsuperscript{203} in order to form blood vessels and deliver more oxygenated blood to the affected area of the body. In biological systems, social behavior is not restricted to cells. Recent work reports on the comparable patterns in the cooperative behavior of bacteria.\textsuperscript{204}

The social behavior of the cells leads to the following assumption: after the realization of the first confluent layer, cells would tend to occupy available space, for example, climb on the vertical wall of the Petri dishes, instead of forming the multilayers of cells, thereby preventing the nutrient deprivation of the first confluent layer of cells at the bottom of the Petri dishes. This was the initial motivation to perform the cell climbing experiments. However, the assumption was not supported by the observation on the wall of the standard polystyrene Petri dishes.
In order to further clarify this hypothesis, in this chapter several experiments are designed to investigate the climbing performance of cells using a variety of materials including polystyrene, silicon, glass and all types of available nanocrystalline diamond samples (smooth to rough as well as hydrogenated, oxygenated, fluorinated and nanocrystalline diamond without any additional surface termination). In the experiments, two different climbing angles (45° and 90°) were applied and three different types of cells (P19, L929, HeLa human cervical cancer cells) were tested, respectively. Moreover, a strawberry patterned diamond sample was also used to test the climbing performance of cells. It is worth noting that climbing cells are new in the literature. Prior to these experiments, climbing was only reported for hematopoietic stem cells moving upwards (5°) under the effect of cytokines released from stroma cells.

The results reveal that cells showed significant climbing behavior on all the tested materials and the best climbing performance on nanocrystalline diamond substrates, with the exception on polystyrene where cells showed no tendency to climb. This indicates that cells had sub-performance on the polystyrene Petri dishes and high performance on the nanocrystalline diamond layers. After a comparative evaluation of the tested materials, it is concluded that the effect of sub-performance of cells on the polystyrene Petri dishes could be due to the interplay of the build-up alkaline pH at the cell-polystyrene interface and/or the change in surface hardness. Finally, nanocrystalline diamond coated Petri dishes are recommended to use for cell culture experiments, especially for sensitive and precious types of cells, such as stem cells, primary cells and embryos.
4.4.1 Cell performance on polystyrene Petri dishes

The tests of climbing ability of cells on the vertical wall of the standard cell culture Petri dishes (polystyrene) were performed by using three different types of cells (P19, HeLa, L929). After the confluent cell layers were formed at the bottom of the Petri dishes, the cells were washed once with PBS, fixed with glutaraldehyde for 20 min at room temperature and subsequently washed twice with PBS. The cells were stained with crystal violet for 30 min at room temperature and followed by careful washing in ultrapure water. When the Petri dishes were dry, the wall of the Petri dishes were separated from the bottom and placed under a light microscope for observation. Figure 4.4.1 demonstrates there was no cell climbing behavior on the wall of the Petri dishes. Indeed the cells at the bottom of the Petri dishes were covered by 1 cm high culture medium, offering a potential climbing height of 1 cm, whereas the cells can only be found at a maximum height of 1 mm on the wall of the Petri dishes. The result was consistent in all the three types of cells.

![Figure 4.4.1 Light microscopy images of cells (P19, HeLa and L929) on the wall of the Petri dishes, presenting no climbing behavior on the wall of Petri dishes.](image)

In order to study cells growth and proliferation on the wall of Petri dish material as well as to facilitate the climbing conditions for cells, the following experiment was
designed: a fraction of the Petri dish wall was positioned at the bottom of a beak and fully covered with culture medium. P19 cells were then seeded on top of the wall of the Petri dish (see Figure 4.4.2a).

The sample was fixed and stained using the above-described method. Light microscopy images present that the P19 cells adhered upon contact on the Petri dish wall during seeding process and proliferated very well during the incubation period, but cells did not climb even with the assistance of the curved structure. As showed in Figure 4.4.2b, no cells were found at the vertical part of the Petri dish wall whereas the rest parts were reaching confluent. This is consistent with the previous observation that cells do not consume or have the energy to migrate against gravity on the wall of the Petri dishes, indicating low cell performance on polystyrene Petri dish material in this specific situation.

![Figure 4.4.2](image)

**Figure 4.4.2** (a) Experimental setup. (b) Photograph and light microscopy images (inset) show Petri dish wall is suitable for cells adhesion and proliferation, but no climbing of cells was observed.

### 4.4.2 Cell performance on nanocrystalline diamond layers

In this section, different materials were employed at two different angles to test the climbing ability of P19 cells. In the first step, nanocrystalline diamond layers with different termination (hydrogen, oxygen, fluorine) and different surface profile,
(average grain size of 15 – 250 nm and root mean square roughness of 18 – 37 nm), as well as silicon wafers were sterilized in 70% ethanol, glued on cover glass and placed at an angle of 45° to the bottom of the cell culture Petri dish (see Figure 4.4.3a). These tested substrates were positioned on the confluent layer of cells directly, or on the plain Petri dish with subsequent cells seeding and incubation until a confluent layer at the bottom of the Petri dish was reached. In both cases, the tested materials were fully covered by cell culture medium and had direct contact with the cells at the bottom of the Petri dish. After that, additional incubation time (26 h) was applied. P19 cells were fixed with glutaraldehyde to induced autofluorescence. Fluorescence microscopy images show that significant cell migration was found on all the tested materials, regardless of the properties of the substrates, such as surface roughness and polarity. The climbing performance of the cells was in general better on diamond than on alternative materials, with the best climbing performance on moderately hydrogenated nanocrystalline diamond. Figure 4.4.3b is a representative image taken on the hydrogenated nanocrystalline diamond substrate at the position of ~ 1 cm (bottom – top) from the bottom of the Petri dish.

**Figure 4.4.3** (a) Experimental setup of cell climbing performance test at an angle of 45° to the bottom of the Petri dish with different nanocrystalline diamond substrates, silicon and glass. Abbreviations are described at the beginning of section 4.1.2. (b) Fluorescence microscopy image of P19 cells on hydrogenated nanocrystalline diamond substrate at a distance of ~ 1 cm from the bottom.
In the second step, the tested materials were located vertically to the cells at the bottom of the Petri dish. Figure 4.4.4 shows four samples (nanocrystalline diamond, polystyrene obtained from bottom of Petri dish, glass, silicon) were vertically mounted on a block processed of polydimethylsiloxane (PDMS), an elastic silicone. After sterilizing in 70% ethanol, the samples and the silicone block were then placed onto a confluent layer of cells, so that all the tested substrates had direct contact with the cells at the bottom of the Petri dish. P19 cells were further incubated for 26 h and then fixed with glutaraldehyde for observation by fluorescence microscopy.

![Figure 4.4.4 Experimental setup of climbing performance test at an angle of 90° to the bottom of the Petri dish with different samples.](image)

Fluorescence microscopy images expose with the exception of polystyrene, the cells climbed on all the test substrates to altitudes close to the height of the cell culture liquid. Figure 4.4.5 displays the result on polystyrene and nanocrystalline diamond substrates. On polystyrene, a few cells reached a height of 1 mm above bottom; on the other three samples, cells climbed significantly higher, showing with 8 mm the best climbing performance on diamond. These experiments validated the hypothesis obtained from the perspective of cells social behavior: instead of forming multilayers at the bottom of the Petri dish the cells prefer to occupy new space for the benefit of
the whole community, even at the cost of the individual cell’s energy – climbing against gravity.

**Figure 4.4.5** Fluorescence microscopy images of P19 cells on substrates placed vertically to the bottom of Petri dish with a confluent cell layer after 26 h incubation. (a) On polystyrene specimen (Petri dish material) the cells climbed to a height of 1 mm. (b) On diamond the cells reached a height of 8 mm.206

Strawberry patterned diamond substrates which were partly immersed in cell culture medium (i.e., tilted with the support of a silicone block, as showed in Figure 4.4.6a) were further used to test the hypothesis. The diamond sample and the silicone block were sterilized in 70% ethanol, put onto a confluent cell layer and incubated for 26 h. P19 cells were fixed with glutaraldehyde and the nuclei were stained with DAPI.

As showed in Figure 4.4.6b, on diamond substrate the climbing was an easy task for the cells. The image shows the flat space between the cavities, occupied by many cells. Cells were able to migrate a distance of more than 1 cm from the bottom of the Petri dish. Interestingly, some cells entered even into the cavities and submerged air bubbles in them.
Figure 4.4.6 (a) Experimental setup of strawberry patterned diamond placed into Petri dish at an angle of 15° to a confluent layer of P19 cells. (b) Fluorescence microscopy image illustrates P19 cells (nuclei in blue) in the space between cavities, ca. 1 cm from bottom. The corresponding position is labeled with blue arrow showed in a.

4.4.3 Discussion

In Petri dishes, the competitive binding between cell-cell attraction and cell-substrate attraction controls the structural formation of cells, for example, for P19 cells they can form embryoid bodies or monolayer of cells. After the completion of the first confluent cell layer, the cells at the periphery of the monolayer have the choice to form a second monolayer on the top of the first one, or to migrate vertically and adhere to the wall of the Petri dish. From the perspective that stem cells and cancer cells are social survivalists, the hypothesis emerges that cells would tend to occupy available surface that would prioritise maximum access to nutrients for all the cells, instead of forming the multilayers. Here this hypothesis is experimentally validated on various substrates with the best and worst results, that is, cell climbing performance, on hydrogenated nanocrystalline diamond and polystyrene Petri dish material, respectively. Here, the question arises why cells do not climb on polystyrene substrate.
Comparing the materials properties of nanocrystalline diamond with polystyrene using the 8 determinants in the biomimetic table, there are four major differences that may be relevant for this issue. First, Young’s modulus of diamond is \( \sim 1200 \text{ GPa} \) and for polystyrene it is much smaller (\( \sim 3\text{GPa} \)). Second, nanocrystalline diamond is distinctive in chemical and biological inertness. Third, nanocrystalline diamond is hydrophobic and polystyrene in the bottom of the cell culture dish is hydrophilic. Fourth, nanocrystalline diamond has constant surface hardness and polystyrene may uptake water during the cell culture process, possibly resulting in changes in surface hardness. In the following text, the four points will be discussed one by one in details.

For the first point, the susceptibility of cells to mechanical variation in their microenvironment is not surprising (see section 4.1.1). For instance, it has been reported in a gradient in stiffness, cells move to stiffer substrates in a process called “durotaxis”.\(^{207}\) However, it is worth noting that the reported 3T3-fibroblast durotaxis was observed only at very low cell densities.\(^{208}\) Moreover, considering the Young’s modulus of cells is only \( \sim 10 \text{ kPa} \), polystyrene and diamond are comparably much stiffer than cells. Therefore, the role of durotaxis in the cell climbing issue can be excluded.

For the second point, diamond is known to be chemically and biologically inert, and do not release any chemicals during the cell culture process. Polystyrene is not toxic in the solid form whereas the monomer styrene is highly toxic.\(^{209,210}\) However, polystyrene cell culture dishes are used routinely in biological research laboratories over the world for almost 50 years.\(^{211}\) To our best knowledge they are fairly biocompatible and can be safely supposed not to release any cytotoxic compounds. Therefore, the possibility that toxic materials are released from the Petri dish material can be excluded.

For the third point, although ion-specific Hofmeister effects for macromolecules in aqueous solution are ubiquitous, the molecular level mechanisms by which ions
operate at the nanointerface are only beginning to be unraveled.\textsuperscript{212} Recent research reveals there is an excess in protons at the water-hydrophobic substrate interface\textsuperscript{213} and by analogy an excess in negative charge (OH\textsuperscript{-}) at the water-hydrophilic substrate interface.\textsuperscript{214} On the former, the tendency is towards acidic; on the latter it is towards alkaline. Since cells are able to sense pH variation, it is plausible to expect that they will also respond to temporal variation in interfacial pH. The increased interfacial pH at the surface of the hydrophilic polystyrene may be decoded by cells as chemical signals (reactive oxygen species), which represents stress and potentially disturbs intercellular communication. This could be the cause for the low cell performance, reflected as no cell climbing.

For the fourth point, experimental results showed that cell climbing behavior was found on other hydrophilic surfaces such silicon, glass and oxygen terminated nanocrystalline diamond, where they have one property in common: constant surface hardness. In the case of hydrophilic polystyrene surface, there may be a change in surface hardness due to uptake of water. It can be a decrease in surface hardness or even an increase, for instance, in the case of porous materials and their reinforcement by water molecules filling the pores. Complementary to change in interfacial pH, cells might register mechanical signals, possibly caused by an associated softening of the surface and/or coupled changes in surface chemistry. We believe that the dimension of the variations is at the nanoscale. However, experimental access to the interfacial pH, or complementary variations, is in no way trivial. An ultra-micro indentation system (Umis-2000, Csio, AU) was used to probe the hardness of polystyrene Petri dishes (Falcon, BD-Biosciences, USA), in dry condition and in previous contact with ultrapure water. Prior to the measurement, Petri dishes were filled with ultrapure water for one day and 14 days respectively and the water was removed before starting the measurements. Probably because of the extended measurement times this approach did not provide significant differences in the depth of indentation, which would indicate a softening of polystyrene. Before the submission of my thesis under
water measurements were hardly realizable with standard equipment. However, afterwards it is approved that the surface of polystyrene Petri dishes became softer when in contact with water by colleagues in our institute using a novel measurement chamber which allows for extended under water nanoindentation experiments.215

Finally, we arrive to interpret the sub-performance of cells on the polystyrene Petri dishes as manifestation of an increased interfacial pH, facilitated by a drop in surface hardness. In other words, the formation of a locally stabilized alkaline zone near the polystyrene surface is likely to represent to cells a signal of reactive oxygen species (ROS), thereby preventing their climbing.

In this study, gravity force is illustrated as an important parameter to explore the cell performance, displaying even minimal differences in the biocompatibility of a material. Therefore, it is suggested to extend the biomimetic table and include gravity force into the list of the determinants.206 In the literature, gravity force has been applied to quantify adhesive interaction between cells. The authors report that hematopoietic progenitor cells actively migrated toward stroma cells against gravity gradient of 5° inclination of the culture plate.205 Importantly, the experimental observations discussed in this section demonstrate that cells on nanocrystalline diamond substrates show higher climbing ability against gravity reflecting high cell performance, in comparison with the suboptimal cell performance on polystyrene Petri dishes. In other words, nanocrystalline diamond substrates show permanent biocompatibility, whereas polystyrene show temporal biocompatibility.
4.4.4 Nanocrystalline diamond coated Petri dishes

Nanocrystalline diamond coated Petri dishes appear to be ideal for important and sophisticated cell culture experiments. The transparent quartz glass Petri dishes with a melting point above 1500 °C were used as the substrates to grow the nanocrystalline diamond layers. At first, the quartz glass Petri dishes were immersed into a solution containing diamond nanoparticles in an ultrasonic bath for 10 min in order to get high nucleation density on the whole surface of the quartz glass homogeneously. Then the nanocrystalline diamond layers were grown on the surface of the quartz glass in the tungsten hot filament CVD reactor. Details of the CVD process were described in chapter 2. Figure 4.4.7a is a photograph of the nanocrystalline diamond Petri dish fabricated in our institute and its nanostructure is revealed by the SEM micrograph (Figure 4.4.7b). The nanocrystalline diamond layers are closed, uniform and perfectly transparent, allowing for their use in the normal light microscopy.

![Figure 4.4.7](image)

Figure 4.4.7 (a) Photograph of a nanocrystalline diamond coated Petri dish with the diameter of 5 cm. (b) SEM image shows the morphology of the nanocrystalline diamond coating at the bottom of the Petri dish.

In the cell culture study, human spermatozoa were incubated on four different types of translucent Petri dishes with the same culture area 19.6 cm²: polystyrene (tissue
culture quality, BD Falcon, USA), quartz glass (QSIL, Germany), quartz glass coated with nanocrystalline diamond and sandblasted quartz glass coated with nanocrystalline diamond. The experimental procedure is as follows. The spermatozoa cells were isolated from fresh human semen by density gradient centrifugation. Washed sperm samples were diluted to a concentration of 4.1 million/ml using Quinn’s sperm washing medium (Sage, USA). The sperm concentration was determined at a temperature of 37 °C using a Makler counting chamber (Sefi Medical Instruments, Israel) mounted on a heated microscope stage. Equal volumes (2.2 ml) of the sperm suspension were seeded into the four different types of sterilized Petri dishes. The Petri dishes were incubated with sperm suspensions for 42 h at 37 °C. Cell vitality was assessed using equal volumes of sperm suspension and eosin solution, as recommended by the WHO for clinical andrology laboratories. Figure 4.4.8a shows the results of the sperm vitality test: The nanocrystalline diamond coated Petri dishes preserved the performance (vitality) of the sperm cells 20% better than polystyrene.

This is consistent with the previous cell climbing experiments (see section 4.4.2): P19 cells show higher cell performance on nanocrystalline diamond than on polystyrene. In section 4.4.3, it is suggested that the sub-performance of cells on the polystyrene Petri dishes may attribute to an increased interfacial pH, which is likely represent to the cells a signal of ROS. Figure 4.4.8b illustrates the origin of the interfacial ROS layer, assumed to induce oxidative stress in both P19 and sperm cells and therefore probably causal for the low cell performance relative to diamond.
Figure 4.4.8 (a) Spermatozoa vitality (live spermatozoa, %) after 42 h incubation on four different types of Petri dishes: polystyrene (PS), quartz glass (QG), sandblasted (DR) treated and untreated (DS) quartz glass coated with diamond. (b) Model explaining the low cell performance (vitality) on polystyrene – the formation of interfacial reactive oxygen species (ROS) layer.217

Indeed, the scientific community has recently expressed the concerns over plastic lab equipment. It is reported that there are chemicals leaching out of the plastic, such as polypropylene pipette tips and tubes, and influencing the results of bioassays.218,219 In view of the broad application of Petri dishes in hundreds of thousands of life science laboratories worldwide, the information emerged from this work may encourage further studies and lead to the development of a qualitative improvement of Petri dishes.220 With additional consideration of the outstanding properties of diamond, nanocrystalline diamond (moderate hydrogen termination) recommends itself to be or to inspire the production of a new generation of cell culture devices, including 2D and 3D Petri dishes and culture flasks, for important cell culture experiments.

4.5 Conclusions

In this chapter, a new definition of biocompatibility was provided by applying the principles of biomimetic thinking. The effort is summarized as the biomimetic table, which provides 8 quantitative determinants describing the physicochemical
environment of a cell as well as an additional determinant: gravity force, using to investigate cell performance. The advantage of the biomimetic table is to analyze each cell’s native environment systematically and to design biomaterials.

The adhesion, proliferation and migration of living cells on nanocrystalline diamond substrates were studied. On the basis of the cell culture observation of the first contact events between cells and diamond substrates, two major biological applications of nanocrystalline diamond layers were developed, where the nanoscopic interfacial water layers play the role of a key determinant. First, strawberry patterned hydrogenated nanocrystalline diamond promises to be a breeding station for embryoid bodies, an important contribution in stem cell research. Here, the hydrogen termination is supposed to be a key element due to its facilitation function in the cell-cell affinity by weakening the cell-substrate affinity, mediated through the highly ordered and stable interfacial water layers on the surface of hydrogenated nanocrystalline diamond substrate. Second, nanocrystalline diamond with moderately hydrogenated is recommended to be a new generation of cell culture devices by showing high cell performance. Here, the interfacial pH on diamond substrate is supposed to be superior for cell performance than that on polystyrene substrate. This issue may have particular relevance on stem cell research, since stem cells preserve their properties in their niche and are sensitive to microenvironment change. The two applications are the immediate fruits of the basic research on interface between materials and biological systems (nanoscopic interfacial water layers), which is described in chapter 3.
5 Nanomedicine

Nanomedicine refers to the medical application of nanotechnology,\textsuperscript{5,221} which brings innovative insights into traditional medical science such as drug delivery system,\textsuperscript{222,223} cancer diagnosis and treatment,\textsuperscript{224,225} and promises to be the future medicine. During the process of investigating nanoscopic interfacial water layers on model hydrophobic and hydrophilic surfaces (chapter 3), a new technique has been developed as a spin-off result, that is, using low-level laser light to manipulate molecular structure of the nanoscopic interfacial water layers. In view that a large portion of intracellular water prevails as interfacial water layers due to the crowded space in the interior of cells,\textsuperscript{26,27} in this chapter I target on physical properties of intracellular nanoscopic interfacial water layers changing in response to moderately intense 670 nm laser irradiation and report new methods in nanomedicine.

In the literature, the biomedical implications of low-level light irradiation (photobiomodulation) in continuous mode in general and with the wavelength of 670 nm in particular have been extensively studied in vitro\textsuperscript{226-228} and in vivo.\textsuperscript{229-233} Section 5.1 gives a brief introduction to photobiomodulation. In section 5.2, cancer cells HeLa are used as a model system to investigate cells’ ability of incorporating cytostatic compounds with and without 670 nm laser irradiation in pulsed mode, respectively. The results demonstrate that the pulsed laser irradiation forces cancer cells to uptake high doses of drugs in a short time. This finding provides strong evidence for a new cell membrane transport mechanism, that is, transmembrane convection, which is induced by the changes of molecular organization of intracellular nanoscopic interfacial water upon 670 nm laser irradiation. The light-cell pump model that is based on the mechanism of transmembrane convection suggests important and unconventional approaches in nanomedicine, such as innovative method in anticancer therapy.
5.1 Introduction to photobiomodulation

Photobiomodulation or low-level light therapy relates to medical applications of light at power levels below that capable of direct tissue change (protein denaturation, water vaporization and tissue ablation). Low-level light irradiation has been shown to modulate a variety of biological processes in vitro and in vivo, ranging from cell proliferation, cell viability to apoptosis. Clinically, 670 nm laser irradiation in continuous mode at the intensity of ~ 1000 W m⁻² has been used for wound healing more than 40 years. Notably, success or failure in photobiomodulation mainly depends on the observation of three basic window parameters: wavelength, irradiance (intensity), and fluence (dose or energy density). In simple words, red to near-infrared light operating in the range of certain fluence and irradiance promotes cell proliferation and viability. The basic Arndt-Schultz curve (Figure 5.1.1) generalizes different modes of cell reaction at different levels of the fluence. When the fluence is in the range of 1 – 4 × 10⁴ J m⁻² (biostimulatory window), the light irradiation increases cell viability significantly, whereas smaller fluence has no observable influence on cells and higher fluence results in the inhibition of cellular functions. Similar threshold exists for the parameter of irradiance, as demonstrated in fibroblasts culture experiments. Importantly, in clinical practice, a forth parameter is no less important than the above-mentioned three window parameters, namely total treatment time. Usually, repeated irradiation is necessary and the extended periods can be ranging from days to weeks with periodic interruptions of 3 days found to provide optimal results.
Figure 5.1.1 Basic Arndt-Schultz curve.\textsuperscript{226}

Although low-level light therapy has been applied clinically for more than 40 years, including but not limited to wound healing, pain relief and soft tissue injury treatment, the mechanism is so far only partially understood. At present, there are two concurrent models to explain the proliferative effect by red to near-infrared irradiation. One proposes that chromophores generate reactive oxygen species in the mitochondria following irradiation.\textsuperscript{236} Whereas excess oxidative stress can harm cells, for instance yielding folic melanocyte apoptosis and DNA damage,\textsuperscript{237} small amount of reactive oxygen species are found to stimulate cell activities. The other paradigm is the production of ATP by the photoacceptor cytochrome c oxidase in the mitochondria.\textsuperscript{238}

This is the state of the art of photobiomodulation in continuous mode. Interestingly, photobiomodulation in pulsed mode is gaining more and more attention but with poor understanding of the effects.\textsuperscript{239-241} Advances in nanotechnology provide possibilities to analyze the phenomena and understand the mechanisms, which will be discussed in the next section.
5.2 The light-cell pump: transmembrane convection

Recent progress in nanotechnology provides important information on the intracellular space. It is densely crowded with numerous macromolecules and organelles, which are predominantly hydrophilic surfaces. Necessarily, a substantial fraction of the intracellular water prevails in the form of interfacial water layers confined between proximal surfaces. Incoherent neutron scattering spectroscopy studies of living cells reveal that the ratio of interfacial water layers compared to total amount of water in cells is ~ 30% and the ratio is reaching much higher values in some organelles, such as mitochondria. In addition, from the laboratory experiments (as described in chapter 3), it is concluded that 670 nm laser light applied at intensity of ~ 1000 W m$^{-2}$ can modulate the structure of interfacial water layers on hydrophilic surfaces that have increased density and viscosity.

In this section, the target is the interaction of moderately intense 670 nm laser light with intracellular nanoscopic interfacial water layers and analogous processes are expected in cells, i.e., a simultaneous reduction of the density and viscosity of the intracellular water prevalent as interfacial water layers. Because of the reciprocal behavior between volume and density, one can expect that upon exposure to moderately intense 670 nm laser irradiation, cells are forced to push out a fraction of their cytosol. This process is facilitated by an increase in the fluidity of the cytosol corresponding to the associated reduction in the viscosity of the nanoscopic interfacial water layers. Conversely, in the dark period following the laser irradiation, the intracellular nanoscopic interfacial water layers consolidate practically instantly, inducing a reflux of water and other small surrounding molecules to the interior of the cells. In other words, the cells are forced to incorporate small molecules, such as water, cytostatic drugs or nutrients, from their surroundings during the light-induced transmembrane reflux process.
This is the basic principle of the light-cell pump model, as illustrated in Figure 5.2.1. The concept of the model was put forward in an earlier study. In this section, the model is validated by using cancer cells HeLa and cytostatic drugs. In the preliminary cell culture experiment, green tea extracted-polyphenols was used as cytostatic drugs, as it is known that the major polyphenol epigallocatechin gallate (EGCG) is a potent tumor inhibitor. Furthermore, the experiments were extended by using two other well-characterized chemotherapeutic drugs: doxorubicin (DOX), methotrexate (MTX), as well as pure EGCG. In the last part of this section, the results are discussed and interpreted. Finally, the light-cell pump model is established with the mechanism of transmembrane convection, operating as an important cell membrane transport method.

**Figure 5.2.1** Illustration of the principle of light-cell pump model. (a) Density and viscosity of intracellular nanoscopic interfacial water layers on hydrophilic surfaces are higher than those of bulk water. (b) Volume expansion based on the modulation of the density and viscosity of intracellular nanoscopic interfacial water layers by 670 nm laser irradiation.
5.2.1 Preliminary experiments with green tea extracted-polyphenols & 670 nm laser light

For the preliminary study, HeLa cells in combination with green tea extracted-polyphenols that are supplied as a cytostatic drug, are used to validate the light-cell pump model. The experimental procedure was as follows. HeLa cells were seeded in 24-well plates, \(1 \times 10^5\) cells in 1 ml cell culture medium per well, and supplemented with 10, 50, 100 µl filter-sterilized (pore size 0.45 µm) green tea solution, which was prepared from 3 g of dry leaf mass per 250 ml ultrapure water, with brewing temperature 100 °C and cooling time 30 min. Subsequent to supplement of the culture medium with the drug, cells were irradiated for 1 min by scanning the line-shaped beam of a 670 nm laser over the wells containing the HeLa cells. The power, intensity, fluence, scanning frequency and beam geometry were 33 mW, 1000 W m\(^{-2}\), \(1 \times 10^4\) J m\(^{-2}\), 1 Hz and 2 × 15 mm\(^2\), respectively. Irradiated cells were incubated with non-irradiated controls for 52 h under standard cell culture conditions, and counted using CASY cell counter.

There are at least four polyphenols in green tea: EGCG, epigallocatechin (EGC), epicatechin gallate (ECG) and epicatechin. EGCG is the most abundant catechin.\(^{243}\) According to the literature,\(^{244}\) the maximum EGCG concentration per well were calculated as 5, 25, 50 µM, respectively. In most in vitro studies EGCG is reported to show a pronounced cytostatic activity at 50 µM.\(^{245}\) The cell culture experiment showed HeLa cells grown at this concentration exposure to the pulsed laser light irradiation resulted in complete proliferation arrest (see dashed line in Figure 5.2.2). Explicitly, in the group of laser light irradiation and 50 µM EGCG, the initial seeding and final cell numbers were virtually equal. In the non-irradiated group exposed to 50 µM EGCG, cell numbers was about three times of the initial seeding numbers, and in the non-irradiated group control group without EGCG, cell numbers was reaching eight times of the initial seeding numbers. In short, moderately intense 670 nm laser
irradiation in pulsed mode was instrumentally in destroying HeLa cells with the presence of EGCG. In the control group without EGCG, the laser light irradiation increased HeLa cells proliferation by 10.1% – in consistence with the basic knowledge of photobiomodulation (see section 5.1).

Figure 5.2.2 HeLa cells proliferation for 52 h incubation. Cells were supplemented with EGCG at different concentrations, with and without periodic irradiation using moderately intense 670 nm laser light. Complete proliferation arrest was realized with 50 µM EGCG and 1 min periodic 670 nm laser irradiation (see dashed line).246

5.2.2 Extended study with well-characterized anticancer compounds & 670 nm laser light

In this section, HeLa cells in combination with three well-defined anticancer compounds, DOX, MTX and EGCG are used to validate the light-cell pump model. The experimental procedure was as follows. HeLa cells were seeded in 24-well plates with $2 \times 10^5$ cells per well at a final volume of 1 ml. Drug concentrations for DOX, MTX and EGCG were 6.4, 400 and 45 µM, respectively, in accordance with previously reported drug-dose data.247 Importantly, all drugs execute their cytostatic
function intracellularly. A description of the related cytostatic mechanisms can be found in the literature.\textsuperscript{247,248} Subsequently, cells were irradiated with the 670 nm laser light according to the aforementioned protocol in section 5.2.1. The well plates containing irradiated cells in drug-supplemented medium and controls were incubated for 3 h under standard cell culture conditions. To assess the effect of the laser light irradiation on the cytostatic potency of the compounds, drug stock solutions were irradiated separately 1 min and added to wells with cell suspensions. The irradiation had no impact on the cytostatic potency of the drugs.

Figure 5.2.3 shows HeLa cell numbers after 3 h incubation. This short period was sufficient to reduce cell numbers in the irradiated groups relative to controls by 23.5\% (DOX), 27.5\% (MTX) and 65.8\% (EGCG). Importantly, the reduction in cell numbers achieved by only drugs were 8.1\% (DOX), 7.0\% (MTX) and 31.3\% (EGCG). In the control group without drugs, there was no difference in cell numbers in irradiated groups versus non-irradiated controls after 3 h incubation. The results are in good agreement with the preliminary study and confirm the validity of the light-cell pump model.

![Figure 5.2.3 HeLa cells proliferation for 3 h incubation. Cells were supplemented with three different cytostatic compounds (DOX, MTX and EGCG), with and without 670 nm laser irradiation, respectively. Statistic analysis was performed with Mann-Whitney U test. All pairings DOX DOX+L, MTX MTX+L and EGCG EGCG+L were significant different (p < 0.01, n=8).](image-url)
In order to visualize the light-cell pump processes, a fluorescent dye (calcein) was used instead of cytostatic compounds. HeLa cells were exposed to a dye concentration of 100 µM and irradiated for 1 min according to the above-mentioned protocol. Controls were only exposed to the dye. Cells were washed four times with PBS and immediately examined by fluorescence microscopy.

It is worth mentioning in contrast to calcein ester, for instance calcein AM, which permeates cell membrane rapidly, underivatized calcein, as used in the present study, enters into cells slowly. Fluorescence microscopy images demonstrate that one minute exposure to pulsed laser light was sufficient to force cells to uptake significant amount of dye molecules, as showed in Figure 5.2.4a. Non-irradiated cells showed no fluorescence, as presented in Figure 5.2.4b. This is a direct visual evidence of the light-cell pump model.

![Figure 5.2.4](image_url) HeLa cells exposed to calcein for 1 min. (a) Laser irradiation during the dye exposure; (b) No laser irradiation.
5.2.3 Discussion

The key to understand the mechanism of the light-cell pump is the structural variation of intracellular water in response to the irradiation of cells with moderately intense 670 nm laser light.250 Normally, the mean density $\rho_c$ of a cell in an equilibrium state is expressed as follows:

$$\rho_c = \frac{m_c}{V_c}$$

where $m_c$ is the cell mass and $V_c$ stands for the space within the cell membrane.

Experiments probing the softness of living cells by recording their response to shear indicated a resemblance to soft materials, such as toothpaste.251 Explicitly, $V_c$ comprises the volume of the water $V_w$ and that of the solid granular material $V_s$ within the cell membrane, i.e., macromolecules and organelles. For more clarity, $V_w$ is defined as the volume of intracellular bulk water $V_{bw}$ and interfacial water layers $V_{iw}$. This is explicitly reflected in the following equations:

$$\rho_c = \frac{m_c}{V_s + V_w} = \frac{m_c}{V_s + V_{bw} + V_{iw}}$$

As described at the beginning of section 5.2, experimental results have demonstrated that a large fraction of intracellular water prevails as interfacial water layers due to the numerous surfaces and interfaces inside of a cell.26-27 In addition, the properties of the interfacial water layers have been explored, presenting with elevated density60,70-72 and viscosity.55,67,68 In this context, considering the spin-off result from the interface study (chapter 3), i.e., moderately intense 670 nm laser irradiation modulates the structure of interfacial water layers thereby inducing a breathing-like volume expansion, one may assume that upon irradiation the cell will either expand its total volume or keep it constant and push out a fraction of its aqueous content. Because expansion involves stretching of the cell membrane, concomitant with its transient
inertia a relatively slow and energy costly process, it is reasonable to expect that a cell with certain permeability to water will favor the second possibility, and regulate the variation in $\rho_c$ by pushing out a fraction of their aqueous content, as illustrated in Figure 5.2.5a. Conversely, during the dark phase, the intracellular interfacial water layers will instantly consolidate, thereby diminishing the intracellular bulk water content. The only possibility left to the cell to compensate for the instant drop in $\rho_c$ is to absorb water molecules from the surrounding environment. Interestingly, small drug molecules that are coincidently close to the cell membrane will be “sucked into” the interior of the cell during the reflux, as illustrated in Figure 5.2.5b. This is a process induced by transmembrane convection, a more efficient transport process than diffusion.

![Figure 5.2.5 Illustration of light-cell pump model with mechanism of transmembrane convection. Moderately intense 670 nm laser irradiation reduces density and viscosity of intracellular nanoscopic interfacial water layers, resulting in the bi-directional flow: (a) expansion (during laser irradiation) and (b) contraction (during dark phase) of the cytosol volume.](image-url)
In general, the transmembrane convection-induced transportation primarily consists of two processes: convective outflow (expansion of the cytosol during light irradiation) and inflow (contraction of water and drug molecules during dark phase). The success of this transport method depends on the drug concentration close to the cell membrane, i.e., within the range of the contractive zone. A probable consequence of the irradiation-induced convective outflow is that drug molecules that are close to the cell membrane are pushed away from the surface of the cell. The question arises: During the dark phase will diffusion be fast enough to bring drug molecules in the cell culture medium back into the range of the convective flow? For clarification, we calculate the average time in which diffusion would transport drug molecules over a test distance of 5 µm. For simplicity, we assume for a drug molecule a radius of 1 nm and calculate the diffusion coefficient $D$ using the Einstein-Stokes equation:

$$D = \frac{kT}{6\pi\eta r}$$

where $k$ is the Boltzmann constant, $T$ is the temperature, $\eta$ the viscosity of the solvent (water at room temperature) and $r$ is the radius of a drug molecule (spherical approximation). Substituting the relevant data we obtain $D$:

$$D = \frac{(1.38 \times 10^{-23} \cdot 300) J}{6\pi (8.9 \times 10^{-4})(1 \times 10^{-9}) \text{ kg s}^{-1}} = 2.5 \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$$

From the Einstein-Smoluchowski equation, relating the mean-square displacement $\bar{x}^2$ to $D$, we obtain the time interval $\Delta t$ required for a drug molecule to overcome a distance of 5 µm:

$$\Delta t = \frac{\bar{x}^2}{2D} = \frac{(5 \times 10^{-6})^2 \text{ m}^2}{2 \cdot 2.5 \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}} = 0.05 \text{ s}$$

The time interval is about one order of magnitude shorter than the dark period of the asymmetric pulse profile resulting from the linear scanning mode (frequency 1 Hz).
and linear beam geometry used in this study. Consequently, in the dark phase, diffusion will be capable of transporting drug molecules pushed away by the convective outflow during the laser light irradiation back into the range of the contraction area.

Notably, for the present system parameters, the diffusion transport is efficient over distances exceeding the typical size of a cell. In this context, another question arises: During the laser light irradiation could convective outflow push the incorporated drug molecules out of the cells? To answer this question it is sufficient to realize that the cytosol is a crowded space. During the relatively long dark phase, diffusion will carry incorporated drug molecules deep inside of the cell. Therefore the relatively smaller water molecules are the most likely candidates to be pushed out of the cells during the laser light irradiation.

In principle, the efficiency of cells’ uptake of drug molecules by transmembrane convection is determined by the interplay between convective and diffusive transport. Three complementary ways are suggested to achieve the optimal results: by extending the irradiation times, by using higher light intensities, and by changing the profile of pulsed light. Therefore light delivery device, such as lasers and light emitting diodes can be adjusted according to the above consideration for further studies.

Additionally, there are several points in this study that need clarification. From the standard knowledge of photobiomodulation, it is clear that the laser light with the parameters employed in this study have proliferative effects on cells if applied in continuous mode. Therefore the possibility of damage in the cell membrane induced by laser light irradiation can be excluded. Moreover, very short incubation time (3 h) were performed, thereby reducing possible contributions from diffusive transport. In summary, it is demonstrated that transmembrane convection induced by pulsed laser light irradiation is a new mechanism for cells to uptake of drug molecules in a short time. These experimental data received recently strong support from the theoretical
side, confirming both properties of interfacial water layers on hydrophilic surfaces: the glue-like character and a higher density compared to bulk water.

This study gains more importance in view that the convective method may overcome the multidrug resistance in cancer cells, which puts limits in cancer chemotherapy. In contrast to the limited pumping capacity of the intrinsic transmembrane efflux pump at the expenses of cellular ATP in cancer cells, the pumping speed and the mass transported per pump cycle in the externally imposed convection are unlimited. Consequently, it is plausible to anticipate the pumping efficiency of the latter is higher than the former, offering a new strategy to overcome the multidrug resistance.

Furthermore, it is important to point out the difference between this transmembrane convective method and photodynamic therapy (PDT). In both methods, identical irradiation parameters are employed, with the exception that the former worked only in pulsed mode and the latter also in continuous mode. Additionally, PDT relies on the limited number of photosensitizes whereas for convective method water is the essential. This aspect should be considered for in vivo applications. Interestingly, according to a recent AFM study, cancer cells isolated from patients were about 70% less stiff than normal cells. This quality was described by Szent Györgyi for malignant tumors. In addition, a transmission electron microscopy (TEM) study showed the number of mitochondria in leukemia cells was significantly higher than that in normal lymphocytes, corresponding to a higher amount of interfacial water layers. This could indicate that the convective method for cancer cells is superior than for normal cells, thus cancer cells can be killed selectively.

Finally, it is worth noting that the transmembrane convection induced by pulsed light irradiation is not limited to cancer therapy. Basically, this method could allow to pump into cells the surrounding small molecules, such as nutrients. This may explain the recent finding in photobiomodulation that moderately intense 670 nm laser irradiation in pulsed mode significantly increased the proliferation and metabolic
activity of HEp-2 cells, as compared to the irradiation in continuous mode, by facilitating the transmembrane transport, pulsed light irradiation forces cells to uptake additional amount of nutrients from the cell culture medium. Apparently the convective mechanism works also with 810 nm light. In other words, the transmembrane convection induced by pulsed light irradiation provides a new method in cell transmembrane transport. This effort supplements the mechanism for low-level light therapy in intermittent mode. Figure 5.2.6 summarizes the synopsis of the basic principles in photobiomodulation. 670 nm laser irradiation operating at the intensity and fluence in the biostimulatory windows in the continuous mode would stimulate cells activity (basic knowledge of photomedicine), whereas in the intermittent mode the irradiation would force cells to suck up the surrounding small molecules (light-cell pump model), thereby destroying or stimulating cells depending on the nature of the molecules.

**Figure 5.2.6** Visual synopsis of the basic principles of photobiomodulation – in continuous and intermittent mode.
5.3 Conclusions

In this chapter, I report the application of the experimental result that moderately intense 670 nm laser light modulates the nanoscopic interfacial water layers (one result from chapter 3), in nanomedicine. The principle of the application is that low-level light irradiation changes the molecular structure and physical properties (density and viscosity) of the intracellular nanoscopic interfacial water layers, which induces transmembrane convection and facilitates the transport of small molecules surrounding the cell membrane into the cells.

Traditionally, there are two mechanisms for the transport of small molecules across cell membrane: facilitated diffusion and active transport. The former depends on the concentration gradient and the latter needs transmembrane proteins (transporters) operating at the expense of ATP. Here, the transmembrane convective method has three significant advantages: on the one hand, it is a much faster process than diffusion, on the other hand, it is not restricted to the transporters, and finally it is externally driven.

The light-cell pump model that is based on the light-induced transmembrane convection has been validated in vitro in HeLa cells using three different anticancer compounds. Considering the potential in vivo applications, transmembrane convection forces cells to incorporate drugs in a short time, thereby protecting healthy cells from extended chemotherapy exposure. Finally, a new method in anticancer therapy is suggested, namely, using the combination of 670 nm laser light and chemotherapeutic drugs.
6 Summary and outlook

The goal of the thesis is to study the biological applications of nanocrystalline diamond layers with the particular focus on the interface between the materials and biological systems. To achieve this aim, nanoscopic interfacial water layers on model surfaces including diamond have been extensively investigated due to their essential roles in biological activities. On this basis, two novel kinds of nanocrystalline diamond based culture devices have been developed. Furthermore, new methods in nanomedicine are inspired by shedding light on the intracellular interfacial water layers.

First part of the thesis deals with new methods on probing structural properties of nanoscopic interfacial water layers prevailing on model surfaces with different polarities in general and hydrogenated nanocrystalline diamond in particular, both in air and under water. Since interfacial water layers are sensitive to observation, information obtained from traditional probing methods with one single tool may be distorted during the observation processes. Hence, a dual technique was applied: one tool was to modulate the structure of the object; the other tool was to monitor the changes simultaneously. As model surfaces, a multitude of biomaterials was utilized, including hydrophobic surfaces (nanocrystalline diamond, hydrogenated nanocrystalline diamond and polystyrene) and hydrophilic surfaces (silicon and gold). Non-thermal laser light with wavelengths that are practically not adsorbed by bulk water, for instance 670 nm at intensity of ~ 1000 W m$^2$, was used to modulate the structure of interfacial water layers, which was imaged by AFAM, QCM and soft XAS, respectively.

AFAM based dual technique allows to probe interfacial water layers on all surfaces with relatively low roughness, but only in air. QCM based dual technique explores interfacial water layers on surfaces both in air and under water – the latter gains importance from the point of view of the biological relevance. More importantly, this
Summary and outlook

The dual technique provides insight into the molecular structure of interfacial water layers and reveals unique information of extraordinary importance. Each variation contributes to partial information, leading to an increasing clear picture of interfacial water layers. Owing to unilateral restriction in mobility, which is induced by the surface, the structure and therefore properties of interfacial water layers differ from those of bulk water. In general, on hydrophobic surfaces, interfacial water molecules are less bounded to the surface and ordered; on hydrophilic surfaces, they are strongly bounded to the surface and present higher viscosity and higher density compared to those of bulk water. The origin of these different properties is the nature of hydrogen bond. It is worth to highlight a spin-off result of the study: using moderately intense 670 nm laser light to influence the hydrogen bond interaction between the interfacial water molecules and thereby modulating the structure of interfacial water layers – the principle of new methods in nanomedicine.

Nanocrystalline diamond is a unique platform to study nanoscopic interfacial water layers initially owing to its extremely chemical inert surface. In this study, it is found that interfacial water layers on hydrogenated nanocrystalline diamond surfaces work as an essential mediator influencing surface properties of diamond under ambient conditions. After analysis and evaluation of a series of electrical conductivity experiments, we arrive at the conclusion that interfacial water layers on hydrogenated diamond surfaces are highly ordered due to the polarization by the hydrogen atoms and bonding to the diamond surfaces. This may help to better understand the mysterious puzzle of electrical conductivity effect on hydrogenated diamond. AFAM
based dual technique reveals that interfacial water molecules on hydrogenated diamond surfaces are tightly bounded to the substrates, probably due to the strong C–H covalent bonding. This finding may explain the phenomenon of extremely low friction between two sliding hydrogenated diamond surfaces. In short, interfacial water layers on hydrogenated nanocrystalline diamond surfaces are highly ordered and stable, which would act as damping layers at the interface between diamond and biological systems.

The second part of the thesis addresses the novel biological applications of the nanocrystalline diamond layers from the aspect of reactions occurring at the interface. Interfacial water layers are listed as the first determinant in the biomimetic table – a new definition of biocompatibility – which provides eight quantitative determinants (nanoscopic interfacial water layers, nanostructure, surface charge, chemistry, microstructure, elasticity, hardness, and biodurability) to describe a cell’s native physiochemical environment, as well as an additional determinant (gravity force) to examine the cell performance. In this thesis, the nanoscopic interfacial water layers have been presented to play a determinant role during the first contact events between cells and biomaterials, and this has been exploited in the long term cell culture experiments.

In the preliminary cell culture experiments investigating the first cell-material contact events, PC12 cells showed significantly higher survival rate on the hydrogenated nanocrystalline diamond layers than that on the non-hydrogenated substrates with the same surface topography. This observation can be explained by using the result from the first part of the thesis: the highly ordered and stable interfacial water layers masking the hydrogenated diamond surfaces behave as protective viscoelastic blankets, thereby ameliorating cells survival rates. The result presented here may suggest using tolerable hydrogenation of conventional implants to increase biointegration.
The biomimetic table is instrumental in designing biomaterials. 3D diamond Petri dish (strawberry patterned hydrogenated nanocrystalline diamond), which are the complete materialization of all the eight physiochemical determinants listed in the biomimetic table, have been applied to culture embryoid bodies from P19 cells. The basic concept for the formation of embryoid bodies is to create conditions where cell-cell attraction is stronger than cell-substrate adhesion. In the case of the strawberry patterned diamond method, the highly ordered and stable interfacial water layers on hydrogenated diamond surfaces facilitate the cell-cell attraction by weakening the cell-substrate adhesion. This method is simple and straightforward, showing significant advantages as compared to the conventional hanging drop method. In view of the importance of embryoid bodies in studying stem cells on the one hand, and strawberry patterned diamond method provides a potential breeding station for embryoid bodies on the other hand, this method represents a big step forward in stem cell research.

A new generation of cell culture devices based on the nanocrystalline diamond layers is recommended since high cell performance has been observed on diamond, as opposed to sub-performance of cells on the standard polystyrene Petri dish material. This results from the designed cell climbing experiments, where gravity force is the key parameter to examine cell performance. After scrutinizing the difference in the surface properties of various materials, it is possible to approach the phenomenon of cell sub-performance on polystyrene substrates as follows: the increased interfacial pH, concomitant with a presumable change in the surface hardness probably supports the formation of a locally stabilized alkaline zone near polystyrene surfaces, which would be sensed by cells as a signal of reactive oxygen species. Consequently, nanocrystalline diamond based cell culture devices may be particularly interesting when culturing sensitive and precious cells, such as stem cells, primary cells and embryos.
The third part of the thesis transfers the spin-off result of the laboratory experiments obtained from the study of nanoscopic interfacial water layers on the diamond model surfaces into the intracellular space, i.e., using moderately intense (~ 1000 W m\(^2\)) levels of 670 nm laser light to modulate the structure of interfacial water layers inside of the living cells. This leads to the establishment of the light-cell pump based on the mechanism of transmembrane convection – a new approach in nanomedicine to transport small molecules into cells. HeLa cells in combination with three different cytostatic compounds (DOX, MTX, and EGCG), have been used to verify this model, which promises a new strategy for anticancer therapy.

In biomaterials science, the study of novel biomedical applications of materials gains benefits from recent advances in engineering science and nanotechnology and further promotes the development of technological innovation – this is clearly reflected in this multidisciplinary work. In particular, the success of this work arises from the unique coincidence of two basic pillars in engineering science and nanotechnology: CVD diamond and AFAM – both present at the Institute of Micro and Nanomaterials. The former allowed to obtain the best possible substrate for the analysis of nanoscopic interfacial water layers; the latter put us in the position to get access to fundamental properties of the nanoscopic interfacial water layers under ambient conditions, and initiated the further experimental measurements including QCM (Institute for Biological Interfaces, KIT) and soft XAS (Functional Materials in Solution, BESSY).

During the process of studying the biological applications of nanocrystalline diamond layers from the perspective of nanoscopic interfacial water layers in the biological systems, many new questions arose, which need further attention. For instance, an important issue in this research concerns the interfacial pH on substrates, in particular on nanocrystalline diamond and standard polystyrene Petri dishes. The technical-biological importance of this complex problem motivates its experimental clarification and may eventually inspire novel methods to approach interfacial pH at the nanoscale. Nevertheless, the results of this in vitro study have already initiated the
research on the application of nanocrystalline diamond based culture dishes in the process of in vitro fertilization (IVF). Further research efforts may extend the cell performance tests addressing functionality in spermatozoa, oocytes and embryos using relevant animal models in order to improve the biocompatibility of Petri dishes used in IVF. In addition, it is noted that there is an interesting phenomenon on hydrogenated nanocrystalline diamond surfaces: hydrophobicity (water hating) at the macroscale and hydrophilicity (water loving) at the nanoscale. Moreover, repetitive application of the light-cell pump model, whose validity has been amply verified in HeLa cells, led to the identification of a promising approach to address one root cause of Alzheimer’s disease: extra- and intracellular depositions of the peptide amyloid-β in the brain. Last but not least the insights derived from biomimetic table, which put us into the position to predict the biocompatibility, at least for in vitro scenarios, are for us a fascinating challenge, which demands for further biological studies. It would be now interesting to design implants with functional surface profiles and coat them homogeneously and durably with the nanocrystalline diamond layers, based on the engineering principles derived from the biomimetic table and to investigate their biocompatibility in pre-clinical trials including long term animal experiments before going over to clinical tests.
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List of publications

Conference contributions


Journal publications

Parts of this thesis have already been published in the following journal articles:
http://science.sciencemag.org/content/318/5855/1424.e-letters
DOI: http://dx.doi.org/10.1557/JMR.2008.0382


The following journal publications are beyond the scope of the thesis:


