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Development of a multifunctional coating system for laser-induced material transport

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ABSTRACT

The aim of our research is to develop a novel surface coating for the use in laser microdissection and laser pressure catapulting (LMPC). LMPC is a contact- and contamination-free technique to separate histologic material and living cells for further proteomic and genomic analysis. Several physico-chemical functions must be included within the optimum coating system designed for this purpose, like optical absorption at the laser wavelength, combined with optical transparency in the visible region, a control of the laser ablation process, mechanical stability and biocompability for the adhesion of the histologic material.

To achieve the optimum system the combination of several layers is required. The optical absorbance to capture the radiation energy from a frequency-tripled Nd:YAG laser ($\lambda = 355$ nm) is reached by a thin layer of zinc oxide (ZnO), deposited by hollow cathode gas flow sputtering. The laser ablation process is controlled by a polyelectrolyte multilayer, consisting of poly(diallyldimethylammonium chloride) (PDADMAC) and poly(sodium 4-styrenesulfonate) (PSS). The evaporation of chemisorbed water from the film is used to promote the catapulting process. For the mechanically stable, laser-dissectible layer organic coatings, like photoresists or lacquers, are suitable. Silica-containing polyacrylate nanocomposites were employed for this purpose.

The investigation of the coating system included LMPC experiments with varying compositions of the layer system. The best results were obtained using a system consisting of ZnO, a polyelectrolyte multilayer deposited from 0.1 M Na_2SO_4 containing polymer solutions, and a 1.5-μm thick layer of the polyacrylate nanocomposite.

To check the quality of the developed system, experiments with the commonly used poly(ethylene naphthalate) (PEN) foil were performed simultaneously. In addition to the determination of the parameters required for LMPC, quantitative real-time Polymerase Chain Reaction (rt-PCR) of the dissected material verified the benefit of the new system.

1. Introduction

The examination of defined samples of histologic material for proteomic and genomic analysis and the separation and transport of living cells is of great interest in many scientific fields like stem cell research, organ culture and tissue engineering. To circumvent mechanical separation techniques, which are tedious, time-consuming and bear the risk of contamination, laser based methods have been developed in recent years [1–3]. A widespread, rapid and contamination-free technique for separation exists in laser microdissection (LMD) of the specimen and subsequent laser-induced forward transport of the material into a vial. The combination of the transport process, called laser pressure catapulting (LPC), and the separation method is often termed "laser microdissection and laser pressure catapulting" (LMPC), which is a rapid, contact- and contamination-free technique. In the late 1990s Schütze and co-workers combined both techniques to isolate minute amounts of biological material [4–6]. The MicroBeam system (P.A.L.M. Microlaser Technologies GmbH), used in the present work, is based on an inverted microscope, but the same principle has been applied for upright microscopes, where the material was transported in opposite direction [7]. The principle of LMPC is illustrated in Fig. 1.

Nowadays the histologic specimens are located on a polymer foil, consisting of poly(ethylene naphthalate) (PEN, 1.3 μm in thickness), which is mounted on a glass slide. The carrier foil is

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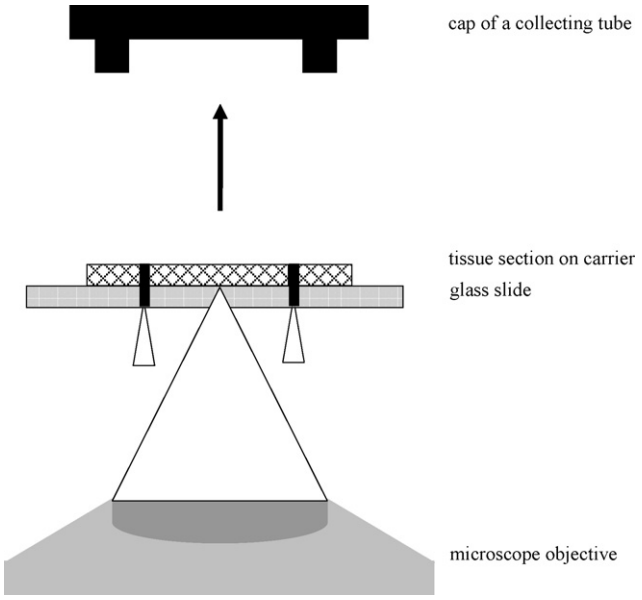

Fig. 1. Principle of separation of small biological objects. A histologic section is placed on the coated glass slide (PEN foil or developed system). The region of interest is laser-dissected with a series of focused laser pulses and afterwards catapulted by a single, more energetic laser pulse into the cap of a microfuge tube.

UV-absorbing, so that the laser light absorption in the specimen is enhanced. Nevertheless, there are several disadvantages inherent to the use of the foil, which should be avoided by the investigated coating system. The optical imaging and the recognizability of the specimens are reduced by scattering and birefringence on the polymer surface. Moreover, insufficient flatness of the foil hampers the experiments and the auto-fluorescence of the foil material makes it impossible to use UV and blue excitation light in immunofluorescence.

The present works deals with the development of a coating system, including several physico-chemical properties, namely:

- optical absorption to capture the laser light,
- an interface layer controlling the laser ablation process and the adhesion of
- a thicker, mechanically stable, UV-absorbing part serving as a kind of laser-dissectible tray to ensure integrity of the cellular material and
- on top a suitable chemical functionality to promote the adhesion of the biological material.

For the LMPC experiments it is very important that the layers are deposited in a specific sequence. The light absorbing layer is located onto the substrate. It is covered with the interface layer which controls the laser ablation process and promotes the ablation of a thicker, mechanically stable, UV-absorbing coating. This tray-like layer may be equipped with chemical functionalities to improve the adhesion of the biological material.

The complete layer system is schematically depicted in Fig. 2. The scheme shows that the demanded properties are realized by using different types of coating.

Optical absorption is achieved by a 100-nm thick layer of zinc oxide (ZnO). Due to its band gap (3.3 eV) and its high thermal conductivity ($60\,W\,m^{-1}\,K^{-1}$, bulk material) [8] it is well suited for the current application. The heat, produced by the laser pulses, is transferred into the adjoining layer, a polyelectrolyte multilayer (PEM) consisting of poly(diallyldimethylammonium chloride) (PDADMAC) and poly(sodium 4-styrenesulfonate) (PSS). The multilayer is deposited via layer-by-layer adsorption from aqueous polymer solutions. The amount of the adsorbed polymer depends significantly on the deposition conditions, like the ionic strength of the solution (concentration of added salt), the salt ion type, the polymers used and the solvent dielectric constant. From the literature it is known, that a certain amount of water is bound in these films by electrostatic interactions [9–11]. In the developed system, the water is evaporated by heat transfer and the expanding volume promotes the ablation process. The histologic material is mounted onto a laser-dissectible, mechanically stable layer, which ensures the integrity of the biological material during catapulting. In the present work this layer consists of a silica-containing polyacrylate nanocomposite.

In contrast to the PEN foil which only adheres at the margins of the glass slide the polyacrylate layer sticks completely to the underlying layer. Hence, an improved flatness of the coating, which facilitates the optical imaging and the recognizability of the specimen, is achieved. Nevertheless, the adhesion of the tray-like coating has to be limited in order to ensure the ablation of specimen areas which are considerably larger than the laser spot. To improve the adhesion of the biological material, the surface can be covered with suitable chemical functional groups.

The developed system differs from the commonly used coatings for LMPC insofar as the layer systems is divided into different functionalities. Moreover, the single components of the system have not been used in ablation processes as yet. One part of the system (ZnO in combination with the polyelectrolyte multilayer) provides a driving force, whereas the second part (the polyacrylate nanocomposite) acts as a tray to prevent the histologic material from mechanical damage. Up to now, water evaporating coatings have to our knowledge not been used for laser-induced material transport.

2. Experimental

2.1. Materials

The substrates used for coating were commercial available glass slides (IDL, D-61130 Nidderau, Germany) which were cleaned with acetone prior to use.

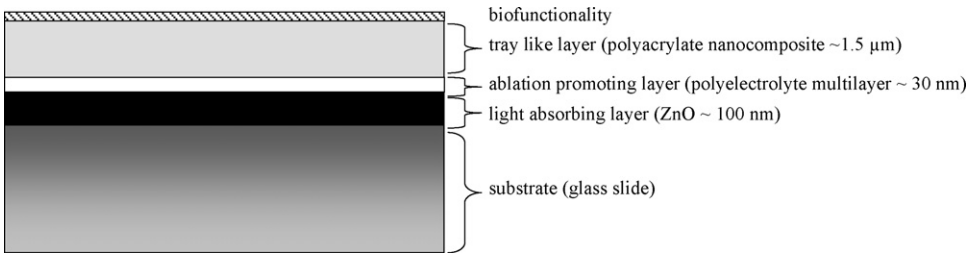

Fig. 2. Multifunctional layer system developed for LMPC.

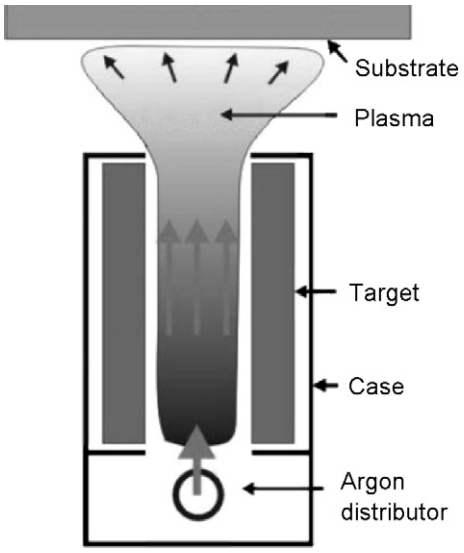

Fig. 3. Principle of gas flow sputtering [12].

Poly(diallyldimethylammonium chloride) (PDADMAC, average M_W ~200,000–350,000, 20 wt% aqueous solution) and poly(sodium 4-styrenesulfonate) (PSS, average M_W ~70,000) were purchased from Sigma–Aldrich. Sodium sulfate and Eusolex® 9020 (4-tert-butyl-4′-methoxydibenzoylmethane) were from VWR (VWR International GmbH, D-64295 Darmstadt, Germany). All aqueous solutions were prepared with deionized water (MicroPure UV, 18.2 MΩ cm) with a pH value of ~7, adjusted using NaOH or HCl, respectively. The polyacrylate nanocomposite was provided by Cetelon Nanotechnik (D-04318 Leipzig, Germany).

2.2. Film deposition

The first layer in the system consists of zinc oxide (ZnO), deposited by hollow cathode gas flow sputtering, prepared in a sputtering system provided by Fraunhofer IST (D-38108 Braunschweig, Germany). The sputtering system consists of a linear gas flow sputter (gfs) source equipped with a zinc target. Fig. 3 illustrates the principle of the method [12]. The process was running at a pressure of 0.3 mbar, with argon and oxygen flows of 0.75 and 0.01 L/min STP, respectively. The substrates were heated to 150 °C prior to film deposition in order to remove the adsorbed water. During the deposition of the oxide the substrates oscillated in front of the gfs source to obtain a uniform coating.

The multilayer of PDADMAC and PSS was adsorbed onto the zinc oxide without any further preconditioning of the surface. The adsorption took place from aqueous solution of the polymers ($c = 10^{-2}$ M, based on the repeating unit molecular weight) containing either no additional salt or 0.1 M Na_2SO_4. For deposition, the glass slides were dipped alternately into the two polymer solutions, starting with the polycation (PDADMAC), for 2 min. To remove the non-adsorbed polymer the substrates were thoroughly rinsed in water after each polymer deposition step, using three different beakers with water (2 min, 2 × 1 min). The process was repeated until 10 double layers (PDADMAC–PSS) were deposited onto the oxide.

The mechanically stable tray layer consists of a polyacrylate nanocomposite, containing nano-sized silica, provided by Cetelon Nanotechnik. The film was deposited by spin coating (1000 rpm, 30 s) and cured photochemically with a mercury lamp (irradiation time, 60 s). After irradiation a thermal curing step was added (45 min, 85 °C hotplate). To induce optical absorption in this layer, 2.5 wt% Eusolex® 9020 was added to the composite prior to spin coating. The enol form of this compound (see Fig. 4) is optically absorbing at λ = 359 nm. The thickness of the film was adjusted to 1.5 μm.

To prove the benefit of the individual layers the structure of the coating system was varied as shown in Table 1.

2.3. Analytical methods

Analyses of the layer system were done by optical absorption spectroscopy, ellipsometric measurements and by profilometry. Moreover the fluorescence of the layer system was investigated and compared with the PEN foil.

UV–vis–NIR transmission measurements were performed using a Cary-5 (Varian, Palo Alto, CA 94304-1030, USA) with a scan rate of 300 nm min^{-1} and a spot diameter of 2 mm. These measurements were accomplished to determine the optical absorbance of each layer.

FTIR-ATR spectra were recorded with a Nicolet 5700 FTIR-spectrometer (Thermo Fisher Scientifc Inc., Waltham, MA, USA) equipped with a MCT detector and a DuraSamplIR single-reflexion 45° diamond ATR crystal using unpolarized light and a spectral resolution of 4 cm^{-1}.

Variable angle spectroscopic ellipsometric measurements were performed with an SE 800 ellipsometer (Sentech Instruments, D-12489 Berlin, Germany) to determine the thickness of the polyelectrolyte multilayers. Single-side polished Si wafers were used as substrates for these measurements and the deposition of ZnO and the PEM system was performed as described earlier. The measurements were performed in a spectral range from 400 to 800 nm, using a Xe-lamp, with angles of incidence between 55° and 75° (step size 5°). Analyses of the spectra were carried out with the software SpectraRay Advanced Fit (Sentech Instruments) for determining the film thickness and the refractive index of the polyelectrolyte multilayer deposited under the described conditions.

To determine the thickness of the polyacrylate layer profilometric measurements were performed after the LMPC. The experiments were carried out with a Dektak 3 (Veeco Instruments Inc., Plainview, NY, USA), equipped with a diamond-stylus (point-size 12.5 μm) and scanning over a range of 600 μm.

For fluorescence measurements the biochip-reader Biodetect® 654/4 provided by Fraunhofer IPM (D-79110 Freiburg, Germany) was used. During the experiment an area of 2 cm^2 was irradiated by a 100-W halogen lamp. The wavelength for excitation and detection of the fluorescence depends on the chosen filters.

2.4. LMPC experiments

The LMPC experiments were carried out with a MicroBeam system (P.A.L.M Microlaser Technologies, D-82347 Bernried, Germany), equipped with a frequency-tripled Nd:YAG laser (λ = 355 nm), where the laser beam is coupled through the beam path for epifluorescence illumination into an inverted microscope (Axiovert 200, Carl Zeiss MicroImaging GmbH, D-37081 Göttingen, Germany). The infinity-corrected microscope objective used was

Table 1
Sample preparation.

Sample	ZnO (nm)	Number of double layers (PDADMAC–PSS)	c, Na_2SO_4 (mol/L)	Energy for laser catapulting (μJ)
#1	100	10	None	2
#2	100	10	0.1	1.4
#3	100	0	None	Not achieved
#4	None	10	0.1	Not achieved

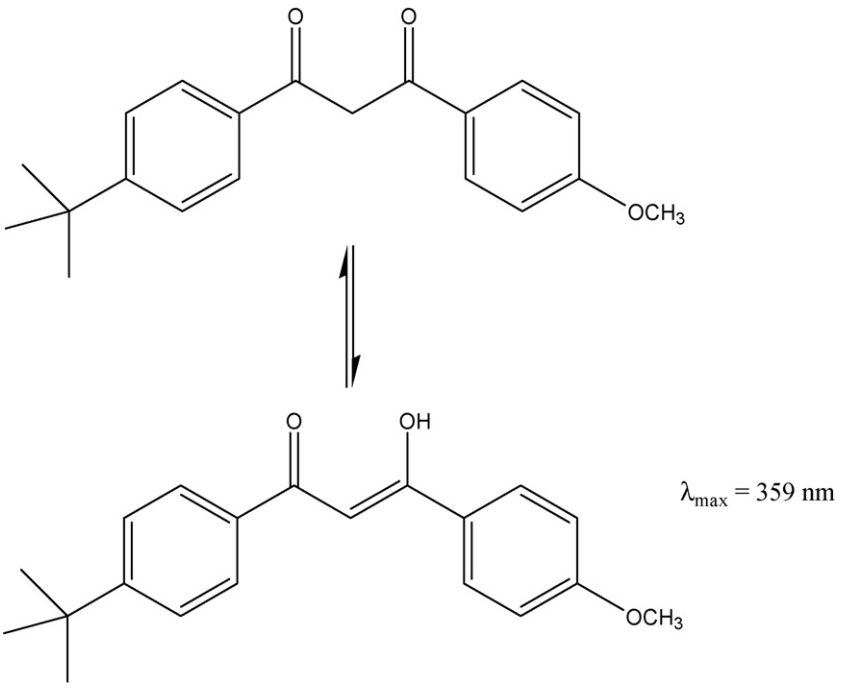

Fig. 4. Keto–enol tautomerism of Eusolex® 9020 (4-tert-butyl-4′-methoxydibenzoylmethane) incorporated into the polyacrylate nanocomposite for UV-A-absorption.

a Zeiss LD Plan Neofluar 20× (NA 0.6). Samples with a diameter of 75 μm were cut with laser energy of 300 nJ. For catapulting the laser was defocused by 30 μm above the specimen and different laser energies were necessary.

If no catapulting was observed up to energies of 9 μJ this sample was considered to fail the experiment.

Molecular biological analyses were performed at the University of Lübeck (Germany). Details are not shown here.

3. Results and discussion

3.1. Preparation of the layer system

The different layers were successfully deposited onto the glass slide as illustrated in the FTIR-ATR spectra (Figs. 5 and 6), which show specific absorption bands of the according compounds. Fig. 5 shows the FTIR-ATR spectra of the polyelectrolyte multilayer of the sample #2. The band at 1470 cm^{-1} is attributed to the asymmetric CH_3 deformation vibrations from the N-bonded methyl groups ($\delta_{as}(N–CH_3)$) in PDADMAC. The absorptions beyond 1600 cm^{-1} are due to C=C stretching vibrations of the aromatic system (ν(C=C)) in PSS. The bands of the sulfonic acid group in this compound, to be expected near 1050, 1250 and 1360 cm^{-1}, cannot be seen in the FTIR-ATR spectra because of the strong absorption of glass, which is still visible in the entire FTIR-ATR spectra (peak not shown), because of the small thickness of the polyelectrolyte multilayer. Chemisorbed water is responsible for the broad absorption at wavenumbers beyond 3000 cm^{-1} (ν(O–H)). In the LMPC experiments the evaporation of bound water from the polyelectrolyte multilayer should act as the major driving force, due to laser energy absorbed in the adjoining, relatively well heat-conducting ZnO.

The PEM deposited under salt-free conditions shows much weaker absorption in the FTIR-spectrum according to the smaller thickness [13–15]. The polyelectrolyte multilayer is a very flex-

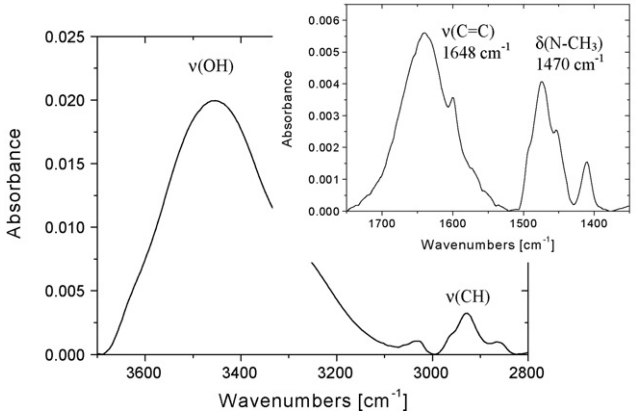

Fig. 5. FTIR-ATR spectrum of the polyelectrolyte multilayer (PDADMAC–PSS, 10 dl, deposited from polymer solutions containing 0.1 M Na_2SO_4).

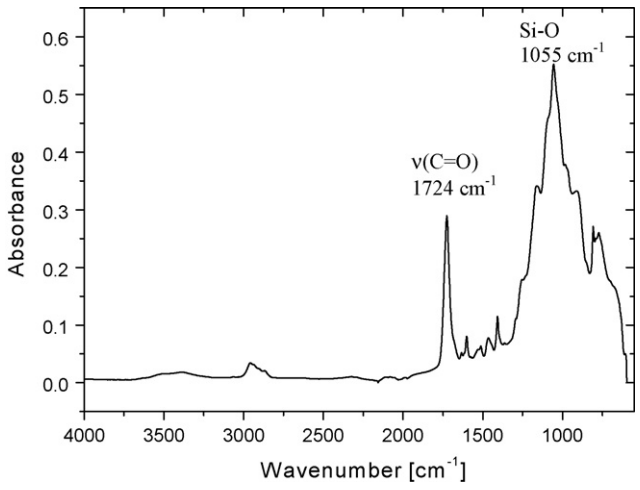

Fig. 6. FTIR-ATR spectrum of the entire layer system.

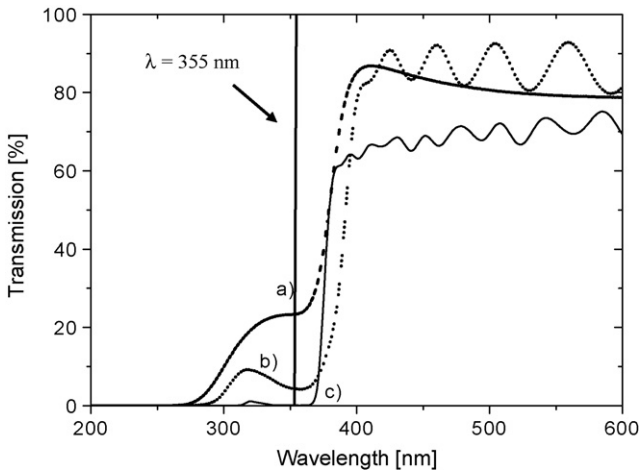

Fig. 7. UV–vis transmission spectra. (a) ZnO (100 nm) on glass; (b) entire layer system; (c) PEN foil.

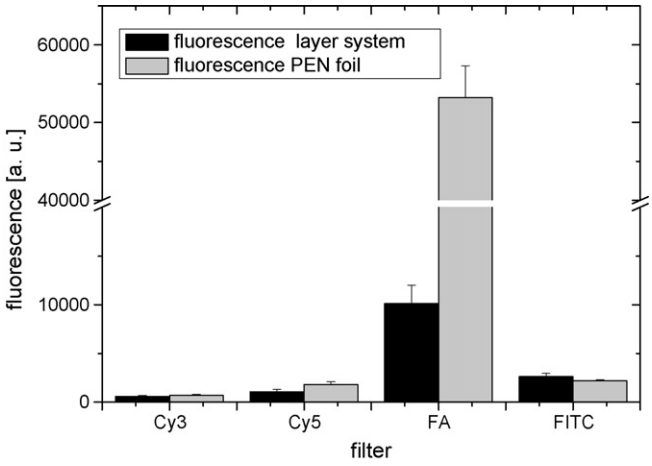

Fig. 8. Comparison of the auto-fluorescence of the polyacrylate nanocomposite and the PEN foil using different filters.

ible system, as the water content or the film thickness can be controlled by the deposition conditions (salt concentration, number of adsorbed layers, pH). Besides, there is a great diversity in the starting material to further control of the film properties. PDADMAC and PSS were chosen, because both are strong polyelectrolytes, which means that the charge density is independent from the pH value. This is very important in the current system, because ZnO is very sensitive to small departures of the solution pH from 7.4 and will dissolve in basic as well as acidic solutions quite well [16].

The polyacrylate nanocomposite was successfully deposited onto the polyelectrolyte multilayer (Fig. 6). The absorption at 1724 cm^{-1} corresponds to the C=O vibrations of the acrylates (ν(C=O)). The strong band at 1055 cm^{-1} is caused by asymmetric Si–O stretching vibrations (ν(Si–O)) attributed to VTMOS.

As the polyacrylate is transparent in the visible region and even non-absorbing at the laser wavelength the incorporation of an UV-A-absorbing compound, like Eusolex® 9020, is necessary.

As shown in the UV–vis-spectra in Fig. 7 the laser light at λ = 355 nm is nearly completely absorbed by the coating. The ZnO layer reduces the incident light to ~22%, this transmission guarantees sufficient energy to be absorbed by the nanocomposite in order to facilitate the microdissection process. The polyelectrolyte multilayer does not absorb at the laser wavelength. A PEN-foil of 1.3 μm in thickness, investigated for comparison, absorbs virtually 100% of the laser light at the wavelength of interest.

However, fluorescence measurements (Fig. 8) illustrate the weakness of the polymer foil with regards to blue and green immunofluorescence techniques. The excitation light path filter for fluorescamine (FA) fluorescence transmits at 395 ± 15 nm, the FA detection filter at 460 ± 20 nm. The common fluorescence dyes FITC, Cy 3 and Cy 5 correspond to the excitation and detection wavelength of these standard markers in immunofluorescence. The polymer foil shows strong fluorescence in the blue spectral region, whereas the investigated system is considered to be non-absorbing.

3.2. Laser microdissection and laser pressure catapulting

The layer system was tested for its suitability in LMPC. In Fig. 9 two samples (diameter 75 μm) are shown, which were laser microdissected and laser pressure catapulted. In both samples a hole in the center of the spot is generated, where even the ZnO is ablated from the surface, which indicates that the energy density of the laser pulse is concentrated there. The fact that ZnO was also removed in this region was proved by profilometry (data not shown). The right image shows an experiment, in which the catapulted specimen landed in the immediate vicinity of the catapulting site. The illustrated examples also belong to the sample #2 (ZnO, 10 dl PDADMAC–PSS and 0.1 M Na_2SO_4), whose coating could be catapulted with the lowest energy. The amount of water and hence the created pressure between the oxide and the nanocomposite plays an important role for catapulting. The evaporated water claims a volume several orders of magnitude greater than the bound water, and the developing pressure acts as a driving force during

Fig. 9. Illustration of the catapulted specimen; laser energy for dissection, 300 nJ; laser energy for ablation, 1.4 μJ; spot diameter, 75 μm.

the catapulting process. A thinner PEM, such as deposited in the sample #1 under salt-free conditions, contains less water and the generated pressure will be smaller. The absence of the PEM (#3) leads to a failure of the ablation process; hence, the important role of water evaporation from the polyelectrolyte multilayer, constituting the major driving force of the process, is demonstrated. Apart from the polyelectrolyte multilayer the ZnO also plays an important role for a successful ablation process, as the water could not be evaporated without it because of inadequate energy absorption by the polyelectrolytes. Nevertheless, caution must be taken during the deposition of the polyelectrolytes, because the oxide is very sensitive to pH values different from neutral. After the coverage with the nanocomposite, however, the ZnO is protected against damage in acidic or basic solutions.

The polyacrylate nanocomposite should act as a tray to prevent mechanical, thermal and photochemical damage of the histologic material. It is important that the material does not break during the catapulting mechanism. Up to now a hole in the center of the catapulted material is formed because of the high energy density, created by the laser pulses. Using coatings with a greater toughness should prevent from damage. Nevertheless, it is possible to catapult an area with a diameter of 75 μm, which is considerably larger than the laser focus. The ablation of the PEN foil requires less energy, but, in contrast to the investigated system the foil, is fixed onto the glass slide only at its margins. The energy responsible for the ablation of the foil does not need to exceed the adhesion to the underlying coating. The polyacrylate coating is non-fluorescent, therefore it is possible to use common immunofluorescence methods. Moreover the layer is transparent in the visible region, so that there are no constrictions, e.g. by inadequate flatness of the coating, by using the microscope. By varying the amount of Eusolex® 9020 the optical absorbance of the organic layer can be controlled. Hence, the ZnO does not absorb the laser light completely; there is sufficient energy, which can be absorbed by the nanocomposite to facilitate the microdissection. Although biofunctionality on the top is still lacking, it is possible to plate the layer system with histologic material. Nevertheless, such a layer is expected to be necessary for the handling of living cells.

First experiments were performed with histologic material, frozen section of a mouse liver, on the coated glass slides. The catapulted tissue was collected in a vial and used for quantitative real-time PCR. The results matched those using the PEN foil.

4. Conclusions

A layer system for the use in LMPC of histologic material has been developed successfully. The demanded properties have been incorporated into the system by designing a multilayer system. So far it is possible to ablate spots with diameters of 75 μm, although the entire layer adheres to the underlying layer and the laser focus is much smaller than the catapulted spot size. The disadvantages of the PEN foil such as insufficient flatness or auto-fluorescence have been avoided. Moreover, it was possible to amplify the RNA from the catapulted tissues and the results correspond to those using the PEN foil.

To prevent the break of the nanocomposite, materials with greater toughness have to be examined. Furthermore, the layer system shall be tested using living cells and further histologic materials.

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