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SU-8 photoresist as material of optical passive components integrated with analytical microsystems for real-time polymerase chain reaction

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Rapid development of analytical microsystems, also often called as labs-on-a-chip and used to perform various chemical and biochemical analysis with nano- and pico-volumes of the sample, has been observed for almost two decades. Successful application of analytical microsystems in medical and veterinary practices is relevant to many factors but one of them is low price of the disposable microsystem – often in the form of a chip. The chips are made of cheap materials, for example, polymers. One of such material is a negative photoresist SU-8. It is characterized by biocompatibility and possibility of easy fabrication of various three-dimensional fluidic micro-structures. Moreover, SU-8 is transparent for visible light. It makes SU-8 attractive material for labs-on-a-chip dedicated for genetic material analysis by real-time polymerase chain reaction (PCR). In this paper, we present the results of investigations of the influence of PCR-like temperature profiling on the transmittance spectra. The autofluorescence effect of SU-8 illuminated with various lasers has also been investigated as one of the factors limiting sensitive fluorescence readout. The results obtained showed that SU-8 can be successfully applied as labs-on-a-chip material but, due to high SU-8 autofluorescence, red-line fluorochromes are preferred when high-sensitivity fluorescence detection is required.

Keywords: SU-8, fluorescence, autofluorescence, microfluidics, lab-on-a-chip, real-time PCR.

1. Introduction

Specialized microdevices for total chemical and biochemical analysis, known as analytical microsystems (μTAS) or labs-on-a-chip (LOC), became important part of the world market of microsystems [1]. Average annual growth rate of the analytical microsystems market amounts to several percentages and is caused mainly by the wide field of applications in life-science, veterinary and medicine. Compared with macroscale devices for analytical chemistry, microfluidic solutions have significant

advantages in terms of speed, throughput, yield, selectivity and control of analyse. However, one of the most important factors deciding on the market success of analytical microsystems is mass-scale fabrication and low-cost per single analytical chip which is the heart of analytical microsystem. In order to reach this goal, simple, cheap and easily scalable to mass-scale fabrication processes and low-cost materials must be applied. Among many materials applied in analytical microsystems technology, polymers seem to be the most commonly used [2–5]. One of them is a negative epoxy--based photoresist SU-8 (Microresist Technology, Germany). The main features of SU-8 photoresist are relatively easy fabrication of high-aspect ratio structures [6, 7] and good biocompatibility [8, 9]. Various microfluidic structures which are parts of the analytical microsystems have been successfully fabricated – microchannels [10] and embedded microchannels [11], micronozzles and microreservoirs [12], as well as microreactors [13]. However, the main advantage of SU-8 is the possibility of fabrication and integration of passive micro-optical elements working in visible light range, for example, lenses, prisms and planar waveguides which co-work with microfluidic components [14–18]. Thus, it is a natural consequence that optical detection methods, especially fluorometry, are also applied in SU-8-based analytical microsystems for molecular diagnostics of genetic material [19], microcytometry of cells and particles or various optical biosensors [15, 17, 19, 20]. One of the most interesting applications of SU-8-based chips is the advanced genetic material analyse by DNA/RNA amplification in polymerase chain reaction process under influence of temperature field with real-time optical biosignals readout (real-time PCR) [21]. The construction of the real-time PCR chip must enable side-wall introduction of the fluorescence light into PCR microchamber by application of an integrated planar waveguide and collection of the fluorescence light transmitted by the upper wall (top cover) of the chip (Fig. 1). This configuration of optical path for fluores-

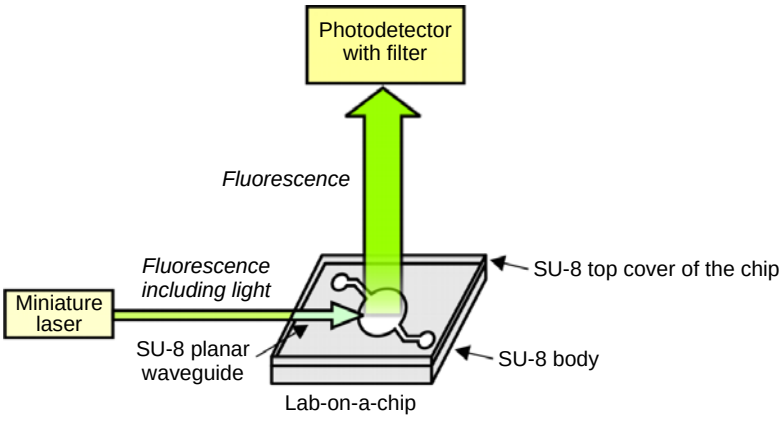

Fig. 1. Scheme of the method of introducing fluorescence inducing light into the PCR microchamber through side-wall planar waveguide and the fluorescence biosignal collection emitted from the LOC made of SU-8.

cence excitation and detection during real-time PCR has been proposed recently by WALCZAK [22].

On the other hand, it is well-known that the lowest detection limit of fluorescence is crucial for real-time PCR analyse. Optical properties of SU-8 important from the point of view of this application limit have not been deeply investigated. Some reports and reviews describe optical properties of this material for room temperature operating labs-on-a-chip [23, 24]. In the case of real-time PCR, two main effects must be considered: the influence of PCR temperature profiling on the SU-8 planar waveguide transmittance and an autofluorescence of SU-8 inducing high background signal limiting high-sensitive fluorescence readout. Although, the autofluorescence of polymers is a well-known phenomenon [25], the autofluorescence of SU-8 has been only briefly described or mentioned [26, 27] without spectral characterization of this phenomenon and time dependencies during laser illumination.

In this paper, we present results of investigations into the influence of PCR-like temperature profiling on transmittance characteristics and the autofluorescence effect during continuous laser illumination, described here for the first time and important for on-chip real-time PCR. The goal of the present work was to find limits of the SU-8 as body material of labs-on-a-chip for real-time PCR. It has been concluded that some effects must be carefully considered when designing and applying analytical microsystems made of SU-8, especially for real-time PCR application.

2. Influence of temperature profiling on visible light transmittance

PCR is a temperature controlled process where three reactions proceed at different temperatures: denaturation at about 94 °C, annealing at 40–60 °C and elongation at about 70 °C. A single PCR cycle contains following three steps mentioned above, each cycle is repeated 30–40 times resulting in significant on-chip amplification of DNA in 30 to 50 minutes. Thus, SU-8 polymer forming body of the chip and planar waveguide is exposed to fast changes of temperature in the range of 40–95 °C.

2.1. SU-8 planar waveguide fabrication

Planar SU-8 waveguide structure has been chosen as a test structure. The core of the waveguide was made of SU-8 2000, whereas cladding was formed by an air surrounding the waveguide from three directions and a glass substrate (Borofloat 3.3, Schott, Germany) on which the waveguide was fabricated (Fig. 2**a**). The shape of waveguides was defined by photolithography step (UV irradiation with 18 mW/cm^2, 30 seconds) with preliminary bake up at 120 °C (6 minutes) and post-exposure bake up at 65 °C (1 minute) and development in SU-8 dedicated developer (6 minutes with stirring agitation). The waveguides were 5 mm, 10 mm or 20 mm long, 200 μm in width and 140 μm in thickness. Endings of the waveguides were finished with the optical fiber ports for assembling 125/62.5 μm glass fibers (Fig. 2**b**). The fibers were glued to the ports by NOA21 UV-hardened glue (Thorlabs, Sweden). One of

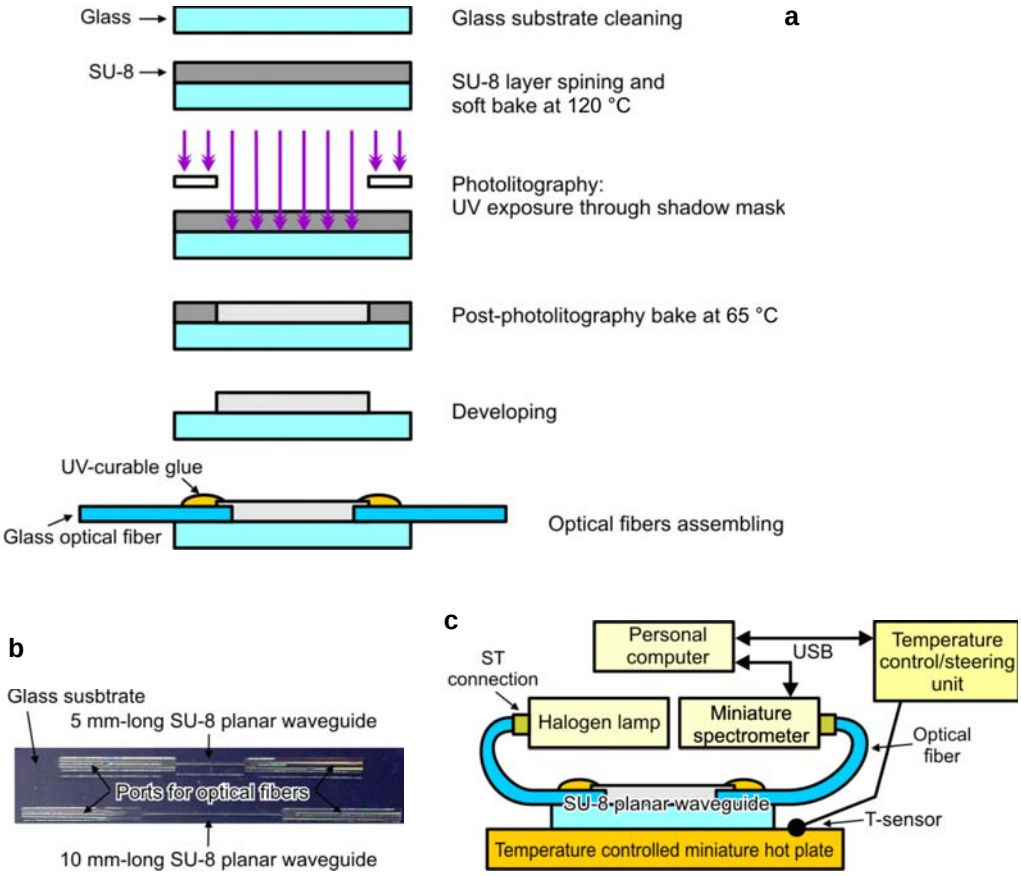

Fig. 2. Transmittance measurement of SU-8 planar waveguide under influence of PCR-like thermoprofiling: fabrication of the test structure of planar waveguide (**a**), view of the planar waveguides prior to the glass optical fibers assembling (**b**), scheme of the measurement set up (**c**).

the fibers was connected to a halogen lamp (Optel, Poland), the second one was connected to a miniature spectrophotometer (Optel, Poland) (Fig. 2**c**).

2.2. Experiment and results

The structure tested was positioned on a computer controlled thermocycler which heated the structure according to the defined PCR-like temperature profile (20 s at 90 °C, 45 s at 60 °C, 30 s at 70 °C). Transmittance measurements were done after the 5th, 15th, 25th, 30th, 40th and 45th temperature cycle. Each of the transmittance characteristics was an average of 5 measurements – averaging procedure was automatically done by the software which was dedicated to the miniature spectrometer.

Transmittance characteristics obtained for a 5 mm long waveguide prior to thermoprofiling and collected at selected PCR-like cycles are shown in Fig. 3**a**. Low

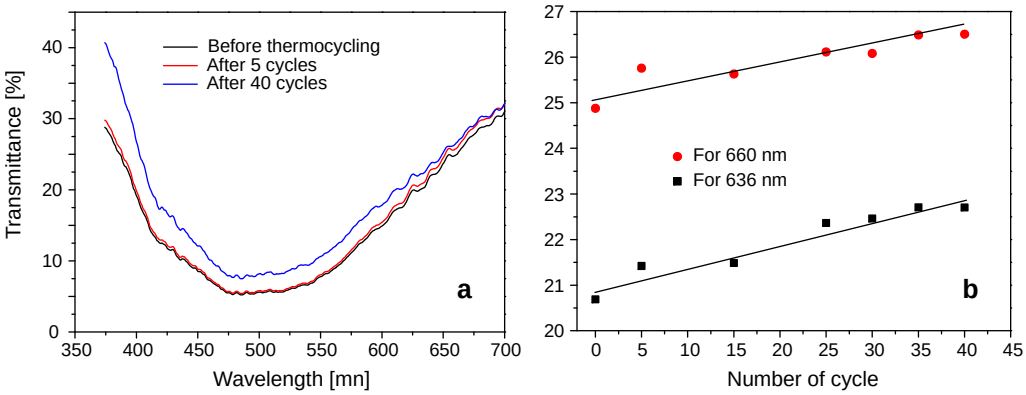

Fig. 3. Transmittance measurement of SU-8 planar waveguide under influence of PCR-like thermoprofiling: spectral characteristics obtained prior and after the 5th and 40th cycles of thermoprofiling (**a**), transmittance cross-sections for 636 nm and 660 nm wavelengths as a function of the PCR-like thermocycles (**b**).

transmittance levels of a 5 mm-long waveguide (around 5 to 20% in the wavelength of interest) seem to be realistic values when compared to the absorption coefficient determined by LEEDS *et al.* [15] (in order of 0.5 cm^{-1}). It must also be pointed out that poor transmittance can be caused by overexposure of the SU-8 layer during photolithographic formation of the structure shape, which was observed by LEEDS *et al.* [15] and during our own work, where UV-light illumination caused significant yellowing of the waveguide structure.

Cross-sectional dependency of the transmittance values for 636 nm and 660 nm were also determined (Fig. 3**b**). As was mentioned earlier, each of the points of these characteristics was an average value of 5 spectral measurements. These two wavelengths were chosen due to the common application of the red-line fluorochromes in real-time PCR (*i.e.*, TO-PRO 3 or Cy5). These fluorochromes are excited at around 635 nm and are flourishing above 650 nm with fluorescence peak in the range 660–670 nm. It has been found that the transmittance values did not change significantly during PCR-like thermocycling (about 2%). Reference measurements done at 21 °C (without thermocycling) showed then during 1.5 hour long measurements, that variations of the transmittance for selected wavelengths were below 0.5%.

The transmittance levels and calculated attenuation of the optical signal obtained for the two wavelengths of interest and for different lengths of waveguides are presented in Tab. 1. Calculation of the attenuation levels was based on transmittance spectral characteristics and basic equation for fiber losses [28]. Calculated attenuation was the sum of the optical signal losses at the interface between optical fiber and the planar SU-8 waveguide (fiber-to-chip interface) and the planar waveguide attenuation itself. The attenuation of the 1 m long glass optical fibers was assumed to be negligible. In order to estimate only the planar waveguide attenuation factor, the cut back method was applied. Thus, estimated attenuation factor for 636 nm was

T a b l e 1. Transmittance and calculated attenuation levels at 636 nm and 660 nm for different lengths of SU-8 planar waveguides before thermocycling; both parameters are the sum of the losses of the SU-8 waveguide and other optical path losses (*i.e.*, fiber-to-chip coupling).

Waveguide length [mm]	Transmittance [%] and attenuation [dB] at 636 nm	Transmittance [%] and attenuation [dB] at 660 nm
5	20.6/6.85	24.9/6.05
10	7.2/11.4	9.1/10.4
20	0.8/20.8	1.5/18.2

around 0.7 dB/mm and for 660 nm 0.6 dB/mm. In this wavelength region, obtained by NATHAN *et al*. the attenuation factor of SU-8 waveguide coupled to a silicon diode was even higher and it was varying from 1.9 to 2.9 dB/mm [29]. On the other hand, attenuation factor reported by LEE *et al*. was around 0.4 dB/mm [20], BORREMAN *et al*. determined attenuation to be around 0.25 dB/mm [30]. Thus, high attenuation is a characteristic feature of SU-8 waveguides. The wide range of attenuation factors reported is probably caused by the application of the various types of SU-8 resists resulting in differences in details of planar waveguide technology and different cladding materials (spinned glass [20], PDMS [15], PMMA [30] or air [29]).

The main conclusion from the point of view of potential application of SU-8 in analytical microsystems for real-time PCR is that no essential influence of the PCR-like thermocycling on transmittance change was observed. However, further test with baking of the planar waveguide at 120 °C caused noticeable yellowing of the test structure and significant decrease of transmittance. It must also be taken into account that parts of the chips which guide the light should be as short as possible due to the low transmittance (high attenuation) in visible light region.

3. Autofluorescence effect

The principle of real-time PCR is real-time collection of the fluorescence light emitted by the fluorochrome which is attached to the double strain DNA. Proper amplification of DNA in the sample analyzed is observed as an increase of the fluorescence intensity. An important parameter describing kinetics of DNA amplification is the cycle number (cycle threshold) when the fluorescence signal intensity enters a logarithmic phase of increase. Cycle threshold C_T can be used to determine initial number of template DNA in the sample analyzed. Thus, more precise and early determination of C_T results in more precise calculation of the template DNA concentration. High optical background signal, coming from autofluorescence of the chip body, makes proper determination of C_T impossible.

A lot of fluorochromes with excitation wavelengths in visible light region can be applied in real-time PCR. Some of them can be effectively excited by laser light with

T a b l e 2. Commonly applied fluorochromes, with excitation wavelengths recommended by the manufacturers and expected emission peaks wavelength; laser operation wavelengths effectively exciting the fluorochromes are also presented.

Fluorochrome (manufacturer)	Family of fluorochromes	Peak excitation [nm]	Peak emission [nm]	Excitation laser [nm]
Fluorescein (POCH)	Blue-line	495	519	488
DyeLight 488 (Pierce Biotech)		493	518	
Alexa Flour 532 (Invitrogen)	Green-line	530	555	532
HEX (Applied Biosystems)		536	549	
TO-PRO 3 (Invitrogen)	Red-line	642	661	636
Cy5 (Amersham Biosciences)		625	670	

fluorescence region shifted towards higher wavelengths (Tab. 2). Unfortunately, these wavelength regions overlap the autofluorescence of SU-8.

3.1. Experiment and results

In the experiments, planar SU-8 waveguides fabricated as previously described and coupled to the glass optical fiber were used. The laser light beam was focused on the surface of the waveguide by the use of an optical microscope objective and positioned close (about 150 μm) to the assembled optical fiber (Fig. 4). The autofluores-

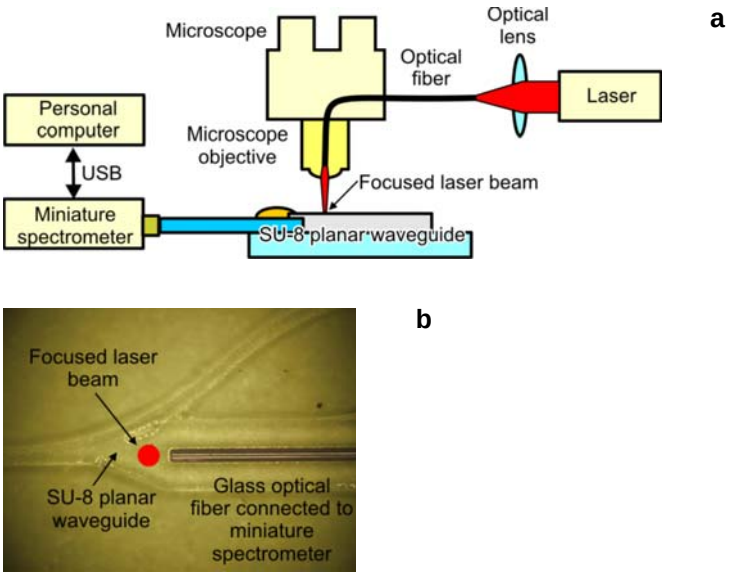

Fig. 4. Measurement of SU-8 autofluorescence: scheme of the measurement set up (**a**), view of the test structure with assembled optical fiber and place of the focused laser beam position marked (**b**).

cence and excitation signals were collected by the fiber and transmitted to the miniature spectrophotometer (Ocean Optics, USA).

Three lasers working at 488 nm, 532 nm and 636 nm with 1.5 mW and 5 mW of optical power were used. During the tests, the SU-8 waveguide was continously illuminated by one of the lasers for 80 minutes, the spectral characteristics of collected light were measured every 5 minutes.

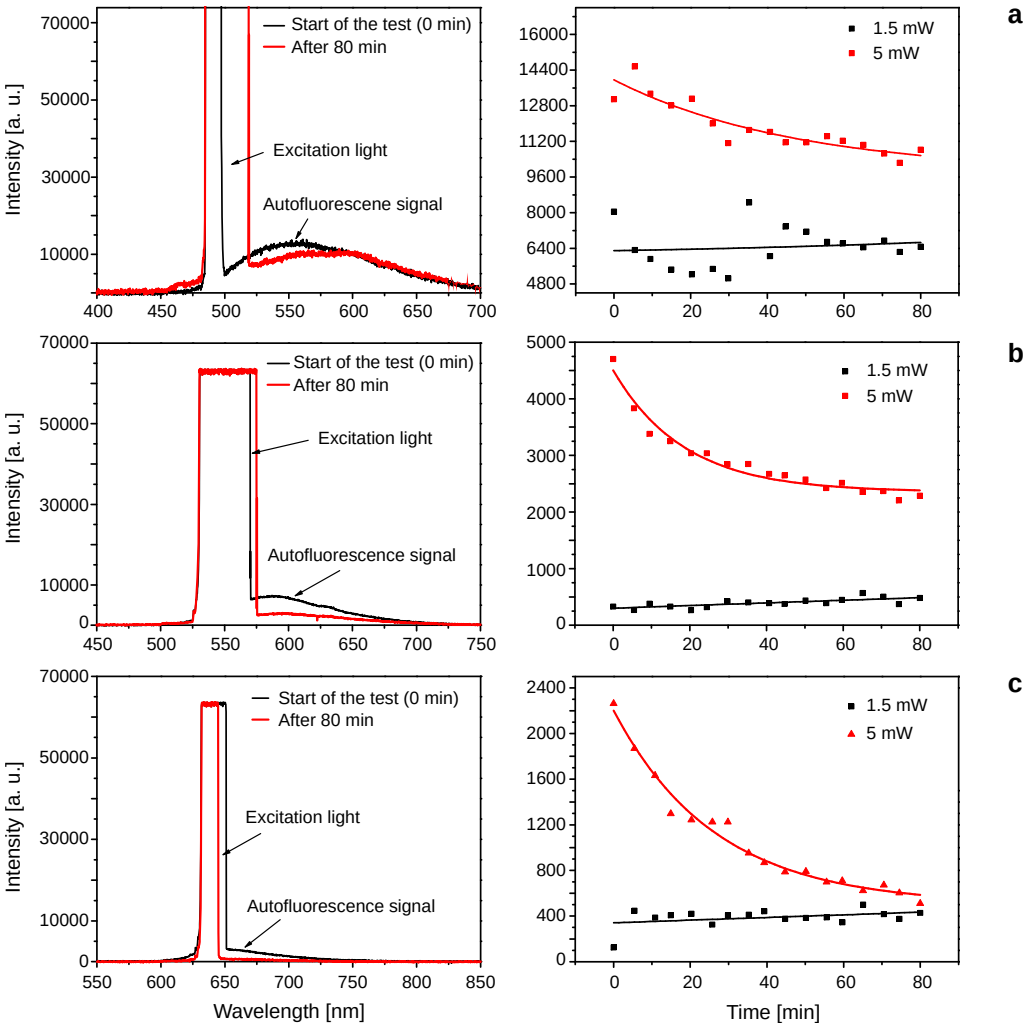

Fig. 5. SU-8 autofluorescence measurements for various laser irradiation wavelengths and powers, left graph shows spectral characteristics of the collected light – applied laser peak (for 5 mW optical power) and autofluorescence signal at the beginning (0 min) and at the end of the illumination (after 80 min) are presented, the right graph is a time dependency of the autofluorescence signal for two laser powers for: laser working at 488 nm, autofluorescence signal extracted at 550 nm (**a**), laser working at 532 nm, autofluorescence signal extracted at 625 nm (**b**), laser working at 636 nm, autofluorescence signal extracted at 670 nm (**c**).

T a b l e 3. SU-8 autofluorescence (AF) intensities for various lasers at constant (5 mW) optical power and transmittance levels for corresponding laser wavelengths

Laser wavelength [nm]	AF intensity [a. u.]/normalized AF intensity	Transmittance at laser wavelength [%]
488	14250/1 at 550 nm	5.5
532	4750/0.33 at 580 nm	7
636	2300/0.156 at 670 nm	20.6

Examples of the spectral characteristics of collected light obtained for lasers applied are shown in Fig. 5. Autofluorescence of SU-8 was observed for all the lasers (Table 3).

It has been found that the autofluorescence intensity decreased during illumination. As expected, the higher power of applied laser induced higher autofluorescence signal. It has also been concluded that longer wavelength of the laser involved caused lower autofluorescence signal (Tab. 3). Decreased autofluorescence for longer wavelengths was also in good correlation with increased transmittance characteristic.

4. Discussion and conclusions

SU-8 negative photoresists is the material which enables relatively easy fabrication of various microfluidic structures with integrated passive optical components (lenses or planar waveguides) forming optical labs-on-a-chip. In this paper, two effects: PCR-like thermocycling and autofluorescence of SU-8 layers have been investigated. Both effects are important from the point of view of application of optical analytical microsystems in DNA analyse by real-time PCR, which is one of the most commonly used molecular diagnostic techniques. It has been found that PCR-like thermocycling did not significantly influence the transmittance characteristics of the planar waveguides. However, temperature exposure above 120 °C should be avoided due to significant yellowing of the structure and rapid increase of the absorbance. Planar waveguides should also be as short as possible due to high attenuation. Therefore, spectrophotometric measurements by the use of microfluidic chip utilizing integrated planar waveguides should be carefully considered. Autofluorescence of SU-8 layer has been clearly observed for all lasers applied (488 nm, 533 nm and 633 nm). For the first time, spectral characteristics of the autofluorescence of SU-8 have been presented. It has been concluded that higher power of applied laser caused higher autofluorescence level. However, the most important conclusion is that longer wavelengths caused lower autofluorescence. Thus, red lasers working in the range of 630 nm to 650 nm are recommended as the fluorescence inducing light sources. This is due to both the high transmittance level and the low autofluorescence of SU-8 layer in this wavelength region.

The absorption of the SU-8 resist layer at wavelength above 300 nm is caused by the absorption of the photoacid generator (PAG) [31]. The aufluorescence of

SU-8 resist excited at the wavelength applied in real-time PCR is caused mainly by the fluorescence of PAG. Due to the fact that the PAG is only in small amounts added to the resist formulation, it can concluded that only a very small amount has strong impact on the autofluorescence intensity. The chemical structure of SU-8 resin, but mainly of the PAG may be the main factor in its autofluorescence. Their large paddle--like shape give the possibility of a large absorbing area which cannot quench their own fluorescence by interference. The use of another PAG in SU-8 could be one alternative to change or reduce the autofluorescence of resist layers.

Another very important fact to consider is the cross linking chemistry of the SU-8 layer. During the exposure to light and the post exposure bake step the PAG generates H^+-ions as it is designed to do. In consequence, the pH is changing and may have a hard effect on the autofluorescence of the SU-8 resist. What is more, the interaction of H^+-ions with a fluorochrome can change delocalization of the electrons present in the conjugated system. This depends upon the fluorochrome and does not mean that every fluorochrome is affected by changes in pH.

Another but small effect on the fluorescence intensity is in general given by temperature. The rising temperature effects are due to deactivation of excited states by collision with other (solvent) molecules. This is a diffusion controlled process and should have in cross linked systems like SU-8 layers only a small or even no impact on the reduction of the fluorescence intensity.

On the basis of investigations described here, novel analytical microsystems for advanced DNA analyse by real-time PCR have been designed and successfully applied under European and Polish projects [32, 33]. Fluorescence induction was realized by a miniature semiconductor laser working at 635 nm, whereas the fluorescence readout was carried out by a CCD minicamera co-working with a 650 nm long-pass filter and developed by us software-based analyse of fluorescence images. Due to minimization of the autofluorescence affect by application of red-line fluorochromes the sensitivity obtained was high enough to observe proper fluorescence signal kinetics during DNA amplification. However, the experimentally confirmed detection limit was an order of magnitude higher than that obtained for lab-on-a-chip made of low-autofluorescence polymer material – cyclic olefin copolymer (COC). The limit of detection for COC

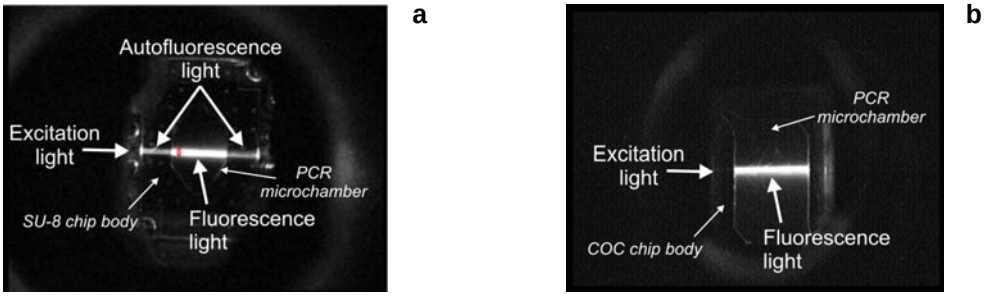

Fig. 6. Views of the SU-8 (**a**) and COC (**b**) labs-on-a-chip dedicated for real-time PCR process with coupled red laser light illuminating the PCR microchamber.

chip was 0.1 ng/ml of DNA for SU-8-based lab-on-a-chip [32] and better than 0.01 ng/ml for COC chip (on the basis of the authors' recent experiments not published yet). In order to illustrate this issue, images of the SU-8 and COC chips done under optical microscope equipped with fluorescence imagining system are shown in Fig. 6. Both chips had the laser light (636 nm, 1 mW) coupled to the PCR microchamber which was filled with DNA and TO-RPO 3 fluorochrome. The fluorescence light should be visible only inside PCR microchambers (as it was for COC chip), whereas, in the case of SU-8 chip, strong autofluorescence was observed within the chip body following propagation path of the laser beam. Strong SU-8 autofluorescence signal from this area was confirmed by spectrometric measurements.

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