Fluorescence Analysis for Amines on Plasma Functionalized Surfaces

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Amino groups on polymer surfaces are exemplary used as anchor groups in combinatorial chemistry for bioassays or microarrays. The surfaces can be polymers like PP or PE where the amines are introduced by an atmospheric pressure dielectric barrier discharge (DBD) microplasma by using silicone-based plasma stamps. The stamps produce functionalized spots with a diameter of 500 µm. Another important application of amino groups is on functional ceramic microtubes for virus filtration. We present methods for the fluorescence analysis of amino groups on these surfaces thereby we show results of measurements from both application fields.

Fluorescence microscopy is advantageous in the examination of area selective plasma-treated surfaces because of the information gained about the amino group densities and especially its uniformity within a single spot, or within a small part of a single spot. Reacting primary and secondary amino groups with fluorescent labels also allows 3D intensity distribution measurements of normally non fluorescent amino groups with sub-micron resolution. With the aid of twophoton-microscopy not only information on the surface of a sample in sub-µm resolution are available like in other analysis methods, but also information on the inner of the substrate can be gained, because of the opportunity of generating 3D images of a stack of collected 2D images. This is especially beneficial during the examination of the treatment depth of the ceramic fibers. The fibers under investigation have an inner diameter of 300 µm.

With the use of specific fluorophores a discrimination of primary and secondary amines is possible. Measuring the fluorescence intensity and the lifetime of a known reference system makes it possible to determine the absolute amount of primary and secondary amines on the surfaces. This will be showed next.

Discrimination of primary and secondary amines

To our knowledge a lot of fluorophores are sensitive to primary amino groups or primary and secondary amino groups. But there is no fluorophore known which is only sensitive to secondary amino groups. That is why we decided to carry out double labeling experiments. In the first step the substrate is labeled with a dye which reacts with the primary amines and thereafter the substrate is labeled with another dye which is sensitive to both, primary and secondary amines. For this method it is important that in the first step all primary amines are labeled with the first dye. Otherwise they will be labeled as secondary amines, because it is later on not possible to distinguish between primary and secondary amines. Another important requirement of these dyes is that they can be exited at the same wavelength, without showing an overlap in their emission spectra. In that case it becomes possible to simultaneously excite and detect them using a dichroic beamsplitter in the emission light and two synchronized detectors. [1], [9] For dyes which are sensitive to primary amines OPA and NDA came into consideration. While the former, OPA, exhibits a bluer emission spectrum resulting in a smaller spectral overlap with other potential fluorophores, the latter, NDA, is more stable.[1],[2],[3],[4] Due to its red shifted emission sulforhodamine 101-sulfonylchloride is chosen as second dye which labels the remaining secondary

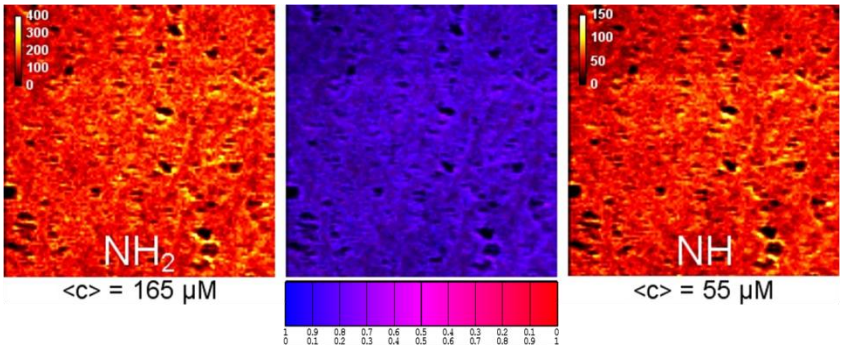
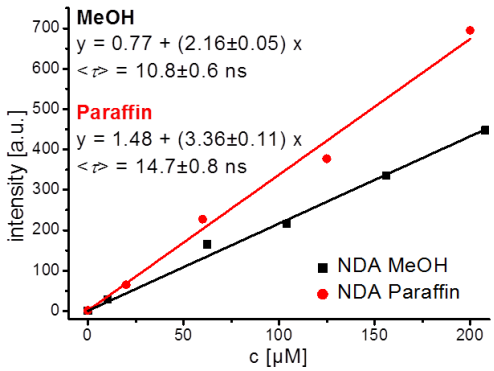


Fig. 1: Concentration distribution of primary and secondary amines on the top side of a plasma functionalized PP membrane after transferring intensities into concentrations via calibration curves [1]

amino groups.[5] One observes either two intensity images one for the primary amines and one for secondary amines or one composite image where both channels are superposed. Figure 1 shows such images after conversion from intensities into concentrations.

Absolute concentrations are obtained by measuring the fluorescence intensities of known amine concentrations in solution in combination with calibration curves. Because of the fact that solutions and surfaces are different systems it is important to measure also the lifetime to get a correction factor for the two different environments to enable a comparison of both. To get authentic results the amines in the reference solution should have the same emission spectra as the labeled amines on the surface. Otherwise they won't be totally collected by the detection system, filters etc. Thus, it is necessary to keep the solvatochromaticity of the solution in order to choose the right solvent. [1], [9] The calibration procedure together with the lifetime correction is exemplary shown for NDA in Figure 2. To avoid under- and oversampling, which could distort the results, it is essential to analyze the reference samples with the same measuring parameters (spatial and time resolution, scanning) as the samples were examined. The amount of amino groups is then obtained as a molar concentration. [1], [9] In order to obtain a comparison to other analytical methods, such as REM-EDX or ATR-FTIR, the concentrations have to be converted into area densities. Table 1 summarizes the results for the PP membrane. PP is available in different densities. To assign the amino group densities to the substrate the porosity of the substrate (density) has to be taken into account. For this reason there are multiple specifications in Table 1, including porous PP and compact PP. Our depth measurements allow the acquisition of information on the amino group densities over the entire treated area. For a plasma-treated BOPP membrane, Klages et al. reported an amino group density of 10 nm^{-2} for primary amines [6]. The data for PP should be nearly the same. Thus, the fluorescence method is a good complementary method to SEM-EDX and ATR-FTIR with a sub-µm resolution and the possibility to make depth measurements for the examination of the whole functionalized area and not only on the outer surface. [1], [9]



$$\underset{y}{\overline{F}} = \underbrace{k\,\Phi}_{m} \cdot \underset{x}{c} + \underbrace{\overline{F_0}}_{b}$$

Φ: not constant, not known

$\Phi \propto \tau$

$$\frac{\Phi_1}{\Phi_2} = \frac{\tau_1}{\tau_2}$$

Fig. 2: Concentration vs. fluorescence intensity calibration curves for NDA and explanation of quantum yield correction via fluorescence lifetime determination [1]

Tab.1: Concentrations, respectively densities of primary and secondary amines [1]

		NH_2	NH	$\sum NH_x$
c_{max}	µM	165	55	220
$c_{max,porous}$	µM	642	215	857
ρ_{max}	nm^{-2}	0.15	0.05	0.20
$\rho_{max,porous}$	nm^{-2}	0.58	0.19	0.77
$\rho r_{complete}$	nm^{-2}	0.45	0.16	0.61
$\rho_{complete,porous}$	nm^{-2}	1.77	0.64	2.41

Not in all cases it is necessary to examine the exact amount of amino groups on a sample. Fluorescence methods using a twophoton-microscope are also advantageous in characterizing the topography and depth functionalization profile of a sample. Because of the sub-µm resolution and the 3D-rendering which was already mentioned above. The next interception deals with amino group imaging on structured surfaces.

Amino Group Imaging on Structured Surfaces

One of the first results during the characterization of porous metal electrode treated substrates is that the depth profile of the functionalization is independently of the plasma treatment time. It is about 1.2 µm for treatment times between 5 and 30 seconds, s. table 2. It can be concluded that the surface roughness is not increased significantly by plasma treatment.

Tab. 2: Fluorescence intensity for different treatment times and widths of Lorentz shaped depth profiles [1]

t s	**<*I*>** a.u.	±	***w*** µm	±
5	540	70	1.1	0.2
10	980	70	1.1	0.8
20	2480	610	1.4	0.3
30	2340	540	1.4	0.4

What can also be seen is that the amino group density increases with a longer treatment time because of higher fluorescence intensities. The left side of figure 3 shows such a rendered 3D image of the fluorescence intensity coming from amines of plasma treated polypropylene membranes. The right side of Figure 3 shows the penetration depth of amines into the substrate during the plasma process where closed cavities were used. As can be seen, here the penetration depth for porous substrates might reach several µm. The extent of depth amination is mainly dependent on the sample porosity [7].

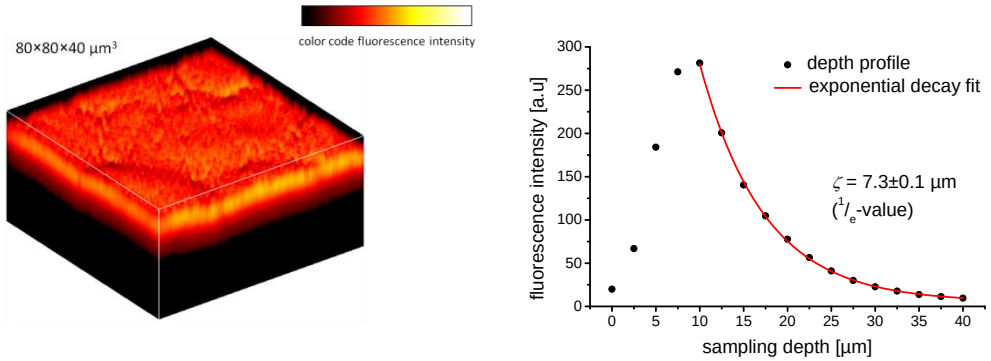


Fig. 3: Rendered 3D image of labeled amino groups after plasma treatment (left) and corresponding fluorescence intensity depth profile (right) with closed cavities setup [1]

As mentioned in the beginning amino groups also play a huge role in the functionalization of ceramic microtubes for sustainable virus filtration. Therefore, it is important to produce defect-free ceramic tubes with pore sizes < 25 nm and a sufficient porosity of about 50.7%.[8] After obtaining such sintered ZrO_2 microtube membranes their functionalization follows stepwise. First the surface is hydroxylated with Piranha solution. The OH- groups make it possible for the amino group containing APTES (3-aminopropyltriethoxysilane) to couple on the surface. The result is an amino-activated membrane which is positively charged under neutral pH conditions. The aim is to restrain the virus once by the pore size as well as their charge. With a pH-shift it is easily possible to clean and regenerate the filter. [8] It is important to get a homogeneous surface with pores forming interpenetrating networks resulting in a large specific surface. A high APTES loading capacity of the membrane increases the positive charge of the membrane and so increases the absorption by charge of the virus. Thus it is important to examine the amine distribution and density on the membrane. Figure 4, left, show a SEM analysis of such a microtube membrane in the overview. In the middle a more detailed view of the membrane is shown where the single pores can be seen. The pore sizes looks very homogeneous over the whole section. The right side of figure 4 shows a fluorescence intensity image of the labelled amines on the surface of the membrane. This picture can be compared very well with the picture in the middle although the resolution is in the µm range. The pore model can also be found in the amine distribution on the membrane. Figure 5 shows a rendered 3D image of labelled amino groups on a microtube membrane of the outer edge of the membrane. It

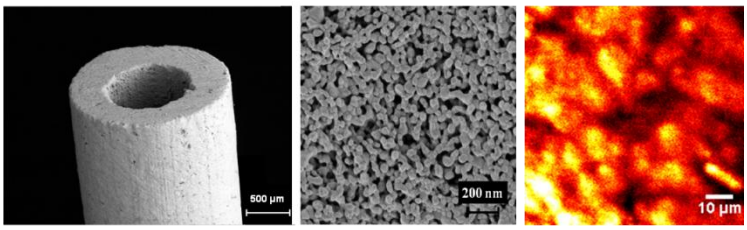


Fig. 4: Left and middle- SEM analysis of a ZrO2 membrane in the overview (left) and in detail (middle)[8], right: Flourescence intensity image of labeled amines on the membrane in µm resolution

gives an overview about the penetration depth of the amines into the pore structure of the tube. The deeper the amino groups penetrate into the tube the deeper the membrane is positively charged and so better is the adsorption of the virus. It also shows the surface roughness of the membrane. The right side of figure 5 shows the related fluorescence intensity depth profile with an exponential decay fit to be able to draw conclusions on the depth of the amino-activated layer of the membrane.

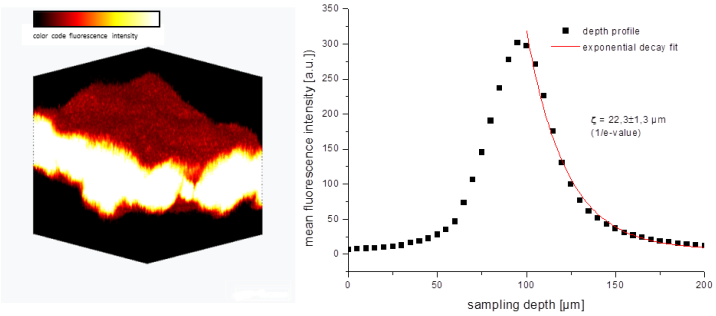


Fig. 5: Left – Rendered 3D image of labeled amino groups on a micro-tube membrane (80x80x205 µm^3), right- corresponding fluorescence intensity depth profile

A single intensity image of a cross section of the membrane can also deliver some rough information on the penetration depth of the amines into the membrane, shown in figure 6 on the left side. A comparison of figures 5 and 6 demonstrates that the highest fluorescence intensity does not lie directly on the membrane surface but rather slightly in the inner of the membrane. Maybe the pores direct on the surface are bigger than the pores in the inner of the membrane and so the pore volume ratio is higher. This would cause less matter in the outer region of the membrane which could be labeled with the amino group sensitive dies. A smaller amount of fluorophores in the vocal volume requires an lower fluorescence intensity.

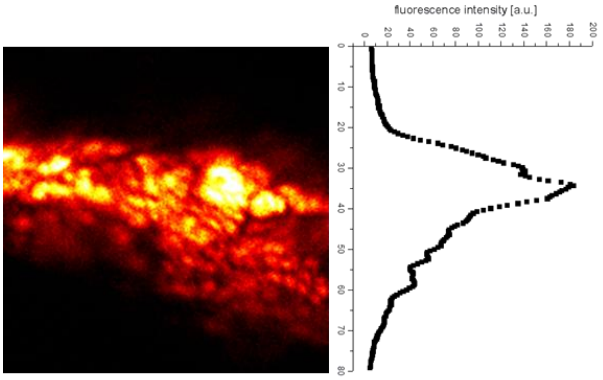


Fig. 6: Left – cross section image of labeled amino groups on a micro-tube membrane (80x80 µm^2), right- corresponding fluorescence intensity profile

Conclusion

We show that two-photon microscopy is especially suited for porous substrates with high sampling depths. With help of fluorescence lifetime measurements by ps-resolved fluorescence recording and suitable references absolute amino group concentrations, respectively densities were determined. Fluorescence anisotropy measurements enable insight into the molecular rotation of fluorescent labels. Altogether, fluorescence analysis provides valuable information about the substrate surface topography, amino group density and uniformity as well as the binding situation with the substrate. All this should help to optimize the production process to receive a homogeneously functionalized substrate.

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