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Quantitative FRAP by means of diffusion through 3D polyelectrolyte shells using confocal and two-photon excitation approaches.

A.Diaspro, D.Mazza, S.Krol, V.Caorsi, P.Bianchini, G.Vicidomini.

MicroScoBio IFOM Research Center, INFM-Department of Physics, University of Genoa, Via Dodecaneso 33, 16146 Genoa, Italy.

In a typical FRAP (fluorescence recovery after photobleaching) experiment, the fluorophores in a region of interest (ROI) are irreversibly "turned off" by photobleaching [1]. Then fluorescence recovery within the ROI, due to the mobility of the marked molecules, is monitored as function of time and fluorescence intensity. The analysis of the fluorescence recovery behavior with a suitable theoretical model allows the evaluation of the diffusion coefficient of the species bound to fluorescent molecules. Unfortunately, the theoretical models reported in literature require precise conditions that often cannot be satisfied by the experimental set-up [1-4]. First, many of the algorithms neglect diffusion processes during the bleach time. Consequently, a sort of "corona" effect arises, resulting in a understimated diffusion coefficient [2]. Unfortunately, this approach works only for homogeneous media (same viscosity in a large area surrounding the chosen region), a condition that often cannot be satisfied when performing FRAP experiments on biological samples. Moreover, due to the spot size of the laser beam, a mismatch between the region chosen and the one effectively bleached is always induced. This phenomenon can cause a significant error when evaluating diffusion processes in structures of a size comparable or smaller than the laser beam spot (~200nm). We are investigating the influence of the "corona effect" as well as the mismatch between the bleaching area and the ROI on the evaluated diffusion. As a heterogeneous model system, a micrometer sized cubic polyelectrolyte capsule with unknown pore width was used [4, 5]. It was through this multilayer system that the diffusion coefficients of different molecular weight dextran molecules were determined. The error induced by the "corona effect" can be eliminated by using the approach described by Weiss [2] for each sample, different FRAP experiments have been performed by varying the bleaching time t_{bleach}. The curves of the estimated diffusion coefficients in dependence of t_{bleach} can be fitted with the empirical function: $D = (a/(1+(t_{bleach}/t_{limit})^b) + c$, where t_{limit} represents the shortest bleach time used. Then, the value of D at $t_{bleach} = \tau_D/15$, for which the corona effect can be neglected, can be extrapolated. Due to the fact that FITC-dex molecules at room temperature diffuse quite fast through capsule walls ($\tau_D/15 \cong 5$ms), the value of D can only be extrapolated to $t_{bleach} = 0$. Then, in order to estimate the error induced by the mismatch of the bleaching spot with the ROI, two different FRAP experiments on cubic polylectrolyte shells have been developed. In the first case, a bleaching region slightly larger than the shell has been chosen, while in the second a region that exactly matches the inner part of the shell was chosen. The results of the measurements performed for the diffusion of 40kDa FITC-dex through the polyelectrolyte multilayer are shown in figure 1. The determined values for the diffusion from extrapolation with the Weiss function [2] are $D_{40kDa, out} = (0.17 \pm 0.07)$ $\mu m^2/s$ and $D_{40kDa, in} = (0.028 \pm 0.008)$ $\mu m^2/s$. The diffusion coefficients measured by bleaching the capsule inside should give a better estimation of the real diffusion through the multi-layered wall than that which relies partly on the bleaching of outside regions. In the second case, also free diffusion plays a role. In case of the linear 10kDa-dextran, the diffusion in presence and in absence of NaCl was

investigated and the values were assessed with $D_{10kDa,\ out}$ = (0.24 ± 0.09) $\mu m^2/s$, and $D_{10kDa,\ NaCl,\ out}$ = (0.12 ± 0.04) $\mu m^2/s$. It was only in the case of inside bleaching that an influence on the diffusion due to the molecular weight of the dextran could be found. The diffusion for 10kDa dextran was extrapolated with $D_{10kDa,\ in}$ = (0.045 ± 0.005) $\mu m^2/s$. Due to the very slow diffusion in presence of a polyelectrolyte shell, the corona outside the capsule should be negligible when the fluorescence was only bleached inside. The results reported here indicate that the matching between the region of interest and the bleached region is crucial to determine the real diffusion coefficient. This aspect can become quite important, especially for structures of dimensions comparable with the laser beam waste size resulting in an overestimation of the diffusion coefficient. Under these conditions, a perspective that can be used to evaluate the error committed by measuring diffusion coefficient could consist in designing a model system that is able to reproduce the characteristics of the studied structure on a larger scale. So far, we propose polyelectrolyte shells as building blocks to study diffusion in complex systems.

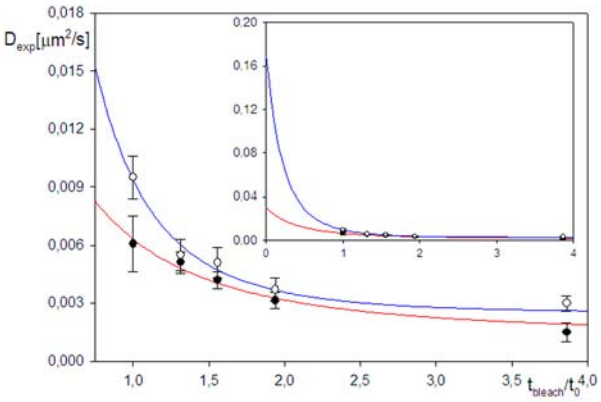

Figure 1: Determined diffusion vs. the bleaching time for FITC-dextran (40kDa) through cubic polyelectrolyte shells consisting of 4 bilayers ($(PAH/PSS)_4$). The lines fit with the equation of Weiss. Graphs for 40kDa FITC-dextran bleached inside the shell (red, filled circles) and a round spot including the shell (blue, open circles). The inset shows the fitting to tbleach = 0 at which the "corona" effect is negligible.

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