Enhanced Green Fluorescent Protein (GFP) fluorescence after polyelectrolyte caging

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Abstract: Discovery of Green Fluorescent Protein (GFP) constituted an important improvement for living cell studies on submicron resolution allowing in vivo fluorescence labeling. We studied the photo-physical properties of single GFP molecules incorporated in a charged polyelectrolyte environment by means of single molecule spectroscopy. The fluorescence characteristics change dramatically in terms of photo-stability, lifetime and blinking behavior so that the proteins scale up to quantum dots. The reported results highlight interesting applications in the design of fluorescent markers and in the development of optical data storage architectures.

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References and links
29. The stabilizing and protecting effect in the polyelectrolyte matrix was also supported by chemically induced denaturation experiments. For this purpose, urea and guanidine hydrochloride were added to the proteins embedded in the polyelectrolyte layers and the disappearance of the fluorescence signals with time was recorded. For both agents a prolongation of the protein unfolding in comparison to wet silica gel-enveloped GFPmut2 was found (data not shown).

1. Introduction
The discovery of the Green Fluorescent Protein (GFP) from the jellyfish Aequorea Victoria has had major impacts on microscopy, live cell imaging, cell biology and research relating to its photophysics [1]. GFP can be expressed by cells as a fusion protein connected to specific cellular structures and spatially visualized by means of three-dimensional optical microscopy methods [2]. Several mutants have been designed to improve or change its photo-physical features, like fluorescence emission (brightness), bleaching stability, photo-activation, photo-switching or pH sensitivity [3-8]. Unfortunately, there remains one major drawback, its low photo-stability [9], even if in some special cases it is possible to recover the fluorescence of the protein after bleaching [10].

In the following we will introduce a new approach in which single GFPmut2 [11] molecules were incorporated into differently charged polyelectrolyte biocompatible matrices, previously used to construct polyelectrolyte shells on living yeast cells [12] or nanogold particles [13]. The photo-stability in terms of photon number released before bleaching and other photo-physical parameters of such a caged GFPmut2 fluorescent protein were measured at single molecule level [14]. Results show a final number of photons released before bleaching only known for fluorescent dyes under vacuum [15] and quantum dots (QDs) in air.
Layer-by-layer (LbL) technique [17] was used to deposit stepwise, in a self-assembly manner, oppositely charged polymers (PSS, poly-(styrenesulfonate sodium salt) and PAH, poly-(allylamine hydrochloride)) on a charged template, forming a sort of capsule [18, 19]. Such a capsule is also used as a container for testing the photophysical properties of GFPmut2 proteins caged in differently charged environments. The motivation for studying GFP photophysics in polyelectrolyte matrices come from the sensitivity of GFP fluorescence dynamics details on the environment. It is known that GFP photophysics is mainly determined by the switching between a neutral (protonated state of the chromophore) and anionic (deprotonated state) state [9, 10]. This balance is largely affected by the environment of GFP. The use of polyelectrolytes to shield GFP from free diffusing charges resulted, as demonstrated in this communication, in a successful method to improve the GFP photo-stability. The results obtained also point out to the potential properties of a new class of fluorescent molecules made by polyelectrolyte encapsulation of single fluorescent proteins.

2. Methods

2.1 Sample preparation

The polyelectrolytes (PEs) poly-(styrenesulfonate sodium salt) (PSS, MW 70 kDa, Aldrich, Milan, Italy) and poly-(allylamine hydrochloride) (PAH, MW 15 kDa, Aldrich, Milan, Italy) were solved in 0.5 M NaCl to a final concentration of 2 mg/mL in Milli-Q grade pure water. As template an amorphous calcium carbonate material with a round appearance (∼ 4.7 μm in diameter) was used. Each PE self-assembly step was performed by incubation of the core in the supersaturated polyion solution (5 min for PAH, 10 min for PSS) followed by centrifugation and two washing steps in 0.5 M NaCl [12]. The encapsulation procedure generally started with PAH as first layer because with zeta-potential the amorphous carbonate material revealed a negative surface charge. Six or eight polyelectrolyte layers were absorbed. Due to the even number of deposited layers the outermost one was always PSS. GFPmut2 is a triple GFP mutant (S65A, V68L, S72A) with enhanced fluorescence emission in comparison to the wild type and an increased protein yield due to a more efficient folding [11].

GFPmut2 proteins were bound to the capsule in three different ways, namely:
1) the amorphous core was incubated for 10 min in the GFPmut2 solution (21 mg amorphous spheres in 0.5 ml 0.5 M NaCl + 10 μl GFPmut2 solution (c(GFPmut2)=1 nM; pH= 6.8)). Then the layer deposition was carried out onto the GFP-doped core;
2) PE capsule preparation was performed like described above and as last step the PSS-terminated capsule was stored for 10 min in a highly diluted GFPmut2 solution (0.5 ml 0.5 M NaCl + 10 μl GFPmut2 solution (c(GFPmut2)=1 nM; pH= 6.8)) attaching the protein with a positive net charge to the outermost layer by means of electrostatic interaction;
3) GFPmut2 was embedded within the PE layer by co-attachment of a GFPmut2-doped PAH solution (0.5 ml PAH solution + 20 μl GFPmut2 solution (c(GFPmut2)=1 nM; pH= 6.8)). For this last configuration one can distinguish two different situations, namely: I) GFPmut2 is embedded in the 3rd layer of a capsule with 6 layers ((PAH/PSS)₃); II) GFPmut2 is enveloped in the 5th layer of a 8 layers capsule ((PAH/PSS)₅).

Additional to the above-described GFPmut2 entrapment into layers on colloidal particles, the single proteins were also investigated in two-dimensional polyelectrolyte layers. In this case, GFPmut2-doped polyelectrolyte solutions (PAH or PSS) were spin-coated on cleaned glass coverslips [20]. Real-time fluorescence images revealed that the initial diffusion of the molecules stops after one hour. Afterwards no diffusion was observable over a 24 h time period.

For all the experiments, GFPmut2 concentration was chosen to be low enough allowing the observation of a reasonable number of single molecules [14, 20].

2.2 Single molecule microscopy

Single molecule fluorescence excitation and detection was achieved by using two-photon excitation utilizing an ultrafast mode-locked Ti:sapphire laser (Tsunami 3960, Spectra...
Physics, CA), (100 fs pulsewidth, 80 MHz repetition frequency) coupled to an inverted microscope (TE300, Nikon, Florence, Italy) (oil immersion objective, 100X, 1.4 NA). Fluorescence emission was selected using a dichroic beam splitter and a band pass filter (515/30) (Chroma Inc., Brattleboro, VT). Two avalanche photodiode (APD) detectors (SPCM-AQ-151, EG & E, Salem, MA) and a three-dimensional nano-piezo stage (Physik Instrumente, Milan, Italy) were used [14]. The acquisition of the fluorescence images (30 x 30 µm) with 1 ms residence time per pixel took around 1 s. The excitation intensity for imaging was 9.5 kW cm⁻² (25 µW). Analysis was performed using a home-coded software allowing photon counting histograms and lifetime pixel-by-pixel computing. A PCI-Board for Time-Correlated Single Photon Counting (TCSPC) (Time Harp 200, PicoQuant, Berlin, Germany) was employed. The analysis of fluorescence profiles was performed by Gaussian fit.

2.3 Single molecule spectroscopy

Fluorescent parameters at single molecule level measurements were carried out through a series of Z-stacks (2.8 µm from the coverslip glass surface) considering the plan, perpendicular to the optical axis, were a maximum section of the nanocapsule is observed. In this way interactions between glass and the fluorophore can be excluded. The bleaching behavior also serves as a marker to distinguish single proteins from clusters. Photo-physical characterization of single GFPmut2 proteins was achieved by measuring the brightness ε, the lifetime, and the bleaching time tBleaching for a single GFPmut2 in the anionic state (λ_{exc}(TPE) = 880 nm, λ_{em} = 508 nm) [14]. Therefore, the fluorescence versus time and the lifetime of single GFPmut2 were collected by focusing the beam laser on a fluorescence spot, i.e. a single protein. The time in which the fluorescence intensity diminishes to background level was defined as bleaching time: t_{bleaching}. The duration for a lifetime measurement, τ, is < t_{bleaching} (typically 10 s at 9.5 kW cm⁻²).

3. Results and discussion

Different sets of experiments have been performed on the caged GFPmut2 proteins. Figure 1 sketches the different caging conditions occurring during the experiments.
The first set of experiments was related to co-adsorption of GFPmut2 with the polycation, PAH (poly-(allylamine hydrochloride)) during the layer self-assembly process onto amorphous calcium carbonate as template. GFPmut2 is mainly located within the specific layer. From the fluorescence features of the detected single molecules one can distinguish three main “internal” configurations, denoted as A\(^{(1)}\), B and C. Apart from co-adsorption steps, capsules, ending with a negative PSS layer, were immersed in a solution containing GFPmut2 proteins. In this case, GFPmut2 molecules attach to the outermost layer and are either in contact to the negative polyelectrolyte or the environmental solution. Two further fluorescent families, i.e., A\(^{(2)}\) and S\(^{(1)}\), can be identified as “external” configuration. As second set of experiments, in order to identify the influence of the polyelectrolyte charge, GFPmut2 proteins were solved in either the polyanion or the polycation solution and spin-coated onto glass substrate. In a third set of experiments, the amorphous calcium carbonate core [19] was doped with GFP or the protein was entrapped in wet silica gel [21]. Two new families were formed, namely: S\(^{(2)}\) in the core and S\(^{(3)}\) in wet silica gel. In all these experiments the proteins were sparsely distributed and single molecule characterization was performed under two-photon excitation regime [14, 22], see Fig. 2.

Fig. 1. GFPmut2 configurations in the capsule A), B) and in spin coated polyelectrolyte layers (red for PSS, blu for PAH). See Methods.
Fig. 2. (Left) GFPmut2 molecules (bright green spots) incorporated in a polyelectrolyte matrix onto amorphous calcium carbonate (dark sphere). The field of view is 3.6 x 3.6 μm (Central) shows the brightness and (Right) is the lifetime image of (Left) panel. So, the left picture is a fluorescence image where each pixel corresponds to the average photons counted per ms. The pixel size of the image of the left panel (50x50 nm) is different from that of the lifetime and brightness images (144 x 144 nm). This enhancement in terms of visualization results in a much blurred image on central and right panels [14].

Fluorescence parameters, for the different families sketched in Fig. 1 and described in the text, are reported in Table 1.

Table 1. Fluorescence parameters for different configurations.¹

<table>
<thead>
<tr>
<th>Configuration</th>
<th>ε [KHz] (1)</th>
<th>1/τ [ns]</th>
<th>T_Bleaching [s] (1)</th>
<th>Quantum yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>External Configuration</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A¹¹</td>
<td>17.0 ± 4.2</td>
<td>3.4 ±0.2</td>
<td>250 ± 18</td>
<td>-</td>
</tr>
<tr>
<td>S¹¹</td>
<td>14.2 ± 3.5</td>
<td>2.8 ±0.2</td>
<td>75 ± 11</td>
<td>-</td>
</tr>
<tr>
<td><strong>Internal Configuration</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A¹¹</td>
<td>16.8 ± 4.1</td>
<td>3.5 ± 0.4</td>
<td>255 ± 13</td>
<td>-</td>
</tr>
<tr>
<td>B</td>
<td>10.8 ± 3.2</td>
<td>2.1 ± 0.3</td>
<td>154 ± 12</td>
<td>-</td>
</tr>
<tr>
<td>C</td>
<td>7.5 ± 2.8</td>
<td>2.5 ± 0.2</td>
<td>108 ± 17</td>
<td>-</td>
</tr>
<tr>
<td><strong>Amorphous Core</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S¹²</td>
<td>14.2 ± 3.3</td>
<td>2.8 ± 0.4</td>
<td>73 ± 5</td>
<td>-</td>
</tr>
<tr>
<td><strong>Wet silica gel</strong> (3)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S¹³</td>
<td>14.0 ± 3.0</td>
<td>2.9 ± 0.2</td>
<td>75 ± 12</td>
<td>33</td>
</tr>
<tr>
<td><strong>Spin coated</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PSS -GFP</td>
<td>16.8 ± 7.0</td>
<td>3.3 ± 0.5</td>
<td>253 ± 19</td>
<td>68</td>
</tr>
<tr>
<td>PAH-GFP</td>
<td>10.7 ± 3.0</td>
<td>2.2 ± 0.3</td>
<td>158 ± 11</td>
<td>39</td>
</tr>
<tr>
<td><strong>Quantum Dots</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>-</td>
<td>150 (4)</td>
<td>19-28 (5)</td>
</tr>
</tbody>
</table>

¹ Notes: (1) - Brightness and bleaching time were determined with an excitation intensity of 9.5 Kw/cm²; (2) - Fluorescence lifetime decay is fitted with two exponential functions: τ₂ = 3.36 ns 50 weight (wt) % and τ₁ = 2.1 ns 50 weight (wt) %; (3) - see [21, 23]; (4) - see [16]; (5) – see [24].

Here, the values of lifetime and brightness are not directly taken on the images. After performing a first fast scan we focus on the each spot, by finding the maximum output, and...
acquire fluorescence versus time in order to record the brightness, its fluctuation, the protein excited state lifetime and the fluorescence blinking up to the bleaching time. The search of the maximum output is performed in few seconds [14]. Usually, about 700 single molecules were used to determine the average values and standard deviations. In order to classify entrapped GFP in families both brightness and bleaching time can be used. However, since bleaching time difference among the families is more pronounced we have used bleaching time to classify the molecules in the different families. The quantum yield values have been measured on bulk samples and not on single molecules. So it was not possible to give a measure of quantum yield on each single protein belonging to the various families. Number of photons released before bleaching as indicator of photo-stability are reported in Fig. 3 for some of the detected families.

![Graph](image)

Fig. 3. Number of collected photons before bleaching versus the excitation intensity. A(0) outside attached to PSS layer (□); A(1) family (●) and B family (O) in the layers; silica gel (S(1)) in table 1 (◊); agarose gel (▼) as in [9].

Variations in excitation energy have a significant effect on the photo-stability: bleaching time under two-photon excitation scales as $I^{-2.5}$ [25, 26]. Nevertheless, with the embedding of proteins in polyelectrolyte matrices as well as using low excitation energy we were able to count up to 9 photons/ms for longer than 9 minutes at 6.6 kW/cm². With about $7 \times 10^6$ collected photons at around 3.8 kW/cm² the photon release of stabilized GFPmut2 is only slightly smaller than the number of photons released by QDs at 20 kW/cm². The dependence of the total photon number detected per molecule scales as $I^{-0.5}$ therefore suggesting that the highest excitation power is not actually the best for fluorescence measurements on single molecules.

Furthermore, the time needed to bleach the GFPmut2 irreversibly, $T_{\text{bleach}}$, was used as a good parameter to quantify the protein stability since it also largely affects the overall brightness of the caged proteins. A single step switch on/switch off mechanism under high-energy excitation serves as a proof that single molecules were measured, see Fig. 4.
A relevant result in terms of photo-stability is that the measured bleaching time for the caged proteins with 4 min is higher than that for CdSe/ZnS QDs in air with 2.5 or 3.5 min [16].

GFPmut2, trapped in gel or in the amorphous carbonate core, exhibits fluorescent properties similar to those of \( S^{(1)} \) family (Table 1). For polyelectrolyte-GFPmut2 spin-coated layers, we observed a more stable species in the PSS environment (PSS/GFP), similar to the \( A^{(1)} \) and \( A^{(2)} \) families related to GFPmut2 molecules trapped within the nanocapsule (Table 1). Moreover, we found a less stable species in the spin coated PAH environment (PAH/GFP) and similar to the species B observed in the nanocapsule layer (Table 1). These similarities point out that family \( A^{(1)} \) can be related to proteins incorporated in the PSS layer. As a proof of concept, the B family for within the polyelectrolyte layer has a fluorescent performance close to the PAH/GFP spin coated ones. For inside the layer, a less stable population, named C, was observed. We assume that this family is populated by proteins trapped in a sort of “nowhere land” between the PAH and the PSS layer.

Comparing the results for GFPmut2 in contact to nanocapsule layers with those of spin coated and uniquely interacting with a single polyion (PAH or PSS) component, the best stabilization was achieved by incorporation in a negative environment. However, also caging in positive polyelectrolyte produced a significant improvement of the fluorescence properties in comparison to wet silica gel or amorphous calcium carbonate entrapment, see Table 1 and Fig. 3. In the last case, it can be assumed that proteins were in water-filled but comparably wide pores with respect to the protein size.

In comparison to QD the caged-GFPmut2 shows some clear advantages. For example, clear spectral differences were observed for the time evolution of QD emission, even for dots of the same batch in the same atmosphere, resulting in a 30 nm blue shift [16]. In the caged-GFPmut2 case, the spectral emission is stable with time. Besides, the number of photons
emitted before bleaching from caged-GFPmut2 under two-photon excitation regime is particularly interesting. It was shown that using lower excitation energy one could prolong the bleaching time. This condition shifted the bleaching time in the range of several minutes. Due to the stabilization of the proteins against irreversible photo-destruction for a long time a high number of photons can be collected in dependence of the excitation energy and of the stabilization method being used as shown in Fig. 3. Another interesting feature is the blinking behavior, Fig. 4, of the GFPmut2 proteins as compared to QDs high frequency blinking [16] that constitutes a problem in some applications. For the caged GFPmut2, at all measured excitation intensities, the maximum blinking frequency, \( v \), is \(<10^{-3} \) Hz, Fig. 4, and the switch-off and switch-on time (\( \Delta_{\text{on}} \) and \( \Delta_{\text{off}} \)) are 950 ± 50 ms and 13 ± 3 min respectively. Because the \( \Delta_{\text{on}} \) and \( v \) have a quadratic dependence on the excitation intensity, we found that at 1.3 kW/cm\(^2\) the \( \Delta_{\text{on}} \) value for the GFPmut2 caged in PSS is about twenty times larger than the one measured for cadmium selenide nanocrystals [16, 24]. Moreover, the quantum yield of the GFPmut2 in PSS and PAH layers resulted larger than the one measured for CdSe/ZnS [24] and for GFPmut2 in gel [4, 21], see Table 1.

Now, the main point of a possible explanation of the results, even if at a speculative stage, can be related to the consideration of the electrostatic interactions between the outer barrel of the GFP and the polyelectrolyte, and of the known enhancement effect of rigidity on the GFP chromophore. We are thinking about a tight wrapping of the polyelectrolyte chains around the protein. Such a charged environment should be responsible for a support and stabilization of the protein structure as well as of the fluorophore inside the \( \beta \)-barrel structure of GFPmut2 [27, 28].

GFP charge distribution (Figs. 5(a) and 5(b)) presents a rim of positive charges (red) around the fluorophore (yellow) [28]. The interaction between these and the negative charges of PSS might induce a tightening and stiffening of the \( \beta \)-barrel near the fluorophore (Fig. 5(c)). This more rigid environment would lead to an increased quantum yield [2] and also to a protection of the protein against bleaching and denaturation [29].

4. Conclusions

Summarizing the results, we conclude that a close wrapping of the green fluorescent protein in polyelectrolyte chains could improve their fluorescence properties (photon release, blinking behavior, photobleaching) reaching an order of magnitude known before only for dyes under
vacuum [15] and for QDs [16]. This points out to the possibility of realizing a new class of fluorescent markers as potential competitors of QDs. The proposed model can also serve for designing more efficient GFP in terms of stability. Moreover, the results reported address a potential application of single caged-GFPmut2 proteins as fluorescent markers in biological systems, based on the high stability in combination with the other photo-physical parameters and with biocompatibility. Interesting developments could also come from the utilization of switchable GFP variants. In this case, the enhanced properties due to polyelectrolyte caging can be exploited in the developments of single-protein based optical memory arrays.

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