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Luminescent Iridium(III)-Containing Block Copolymers: Self-Assembly into Biotin-Labeled Micelles for Biodetection Assays

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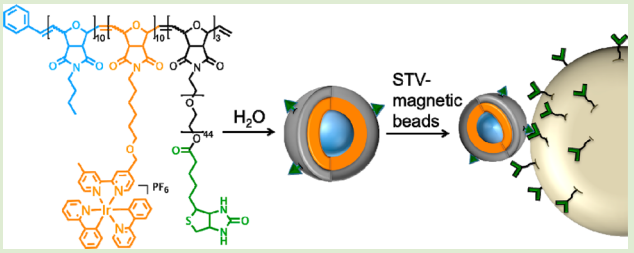
Supporting Information

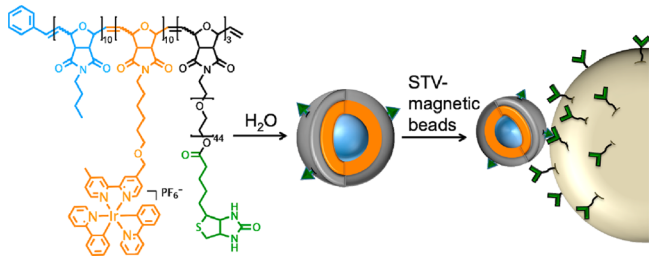
ABSTRACT: Luminescent polymers containing $Ir(ppy)_2(bpy)PF_6$ complexes, biocompatible poly(ethylene glycol) (PEG) chains, and biotin moieties were synthesized via ring-opening metathesis polymerization (ROMP). Their self-assembly in water into micelles resulted in an increased quantum yield compared to open polymer chains in acetonitrile, which is likely due to core rigidity and desolvation. Streptavidin coated magnetic beads were employed to analyze the binding ability of these micelles. The positioning of the molecular recognition moiety biotin within the polymer chain had a very significant effect on the availability of biotin on the micelle surface and the ability of the micelles to bind to streptavidin. Simply attaching biotin to the end of the ROMP polymer yielded micelles in which the biotin units were shielded by the PEG chains, whereas the synthesis of a new ROMP monomer containing biotin at the end of the PEG chains resulted in improved surface availability of the biotin group. Preliminary experiments in which streptavidin was microcontact-printed onto functionalized glass coverslips also indicated specific binding between the micelles and streptavidin and further demonstrated the potential of these micelle systems to function as luminescent probes in solid-phase biodetection assays.



Many assays for the detection of biomolecules utilize probes labeled with luminescent markers to detect increasingly small quantities of analyte. This approach requires methods to amplify the analyte, such as the polymerase chain reaction for nucleic acids, but such methods can be labor-intensive, can lead to loss of some information, and are not readily applicable to protein detection.[1] To increase the sensitivity of assays and to sidestep difficulties in analyte enhancement, methods to amplify the signal itself, rather than replicate the analyte, are desirable.[1,2] A particularly attractive approach to achieve this would be to replace the standard fluorescent probe in a biodetection assay with a polymeric nanosphere containing multiple luminescent centers.[3]

The self-assembly of amphiphilic block copolymers can result in well-defined micellar aggregates, thus providing a facile method of building these polymer nanospheres.[4] The judicious selection of monomers for these block copolymers is required to impart both robust assembling properties and selective biomolecule binding. First, a hydrophobic block is needed to induce self-assembly and will comprise the micelle core (Scheme 1). Second, a water-soluble and biocompatible block that prevents nonspecific binding is necessary to form the corona.

Scheme 1. Self-Assembly and Streptavidin Binding of Biotin-Labelled Micelles



Third, the polymer must contain a large number of luminescent moieties as part of its structure. Luminescent iridium(III) complexes are excellent candidates, as their large Stokes shifts make them less prone to self-quenching than organic fluorophores.[5] In particular, they are widely studied for biodetection assays because their emission is tunable over a wide range of wavelengths with good quantum yields, they are

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resistant to photobleaching, and they possess long luminescence lifetimes.[6] Many of these complexes have been shown to be efficient in designs utilizing electrochemiluminescence, thus providing additional mechanisms for signal enhancement,[3g,7] and a number of light emitting Ir(III)-containing polymers have been generated.[8] Finally, the micelles must have a biorecognition element displayed on the micelle surface that is able to bind to target groups. This binding may be hindered by the micelle structural characteristics, and careful polymer and micelle design is required.[9]

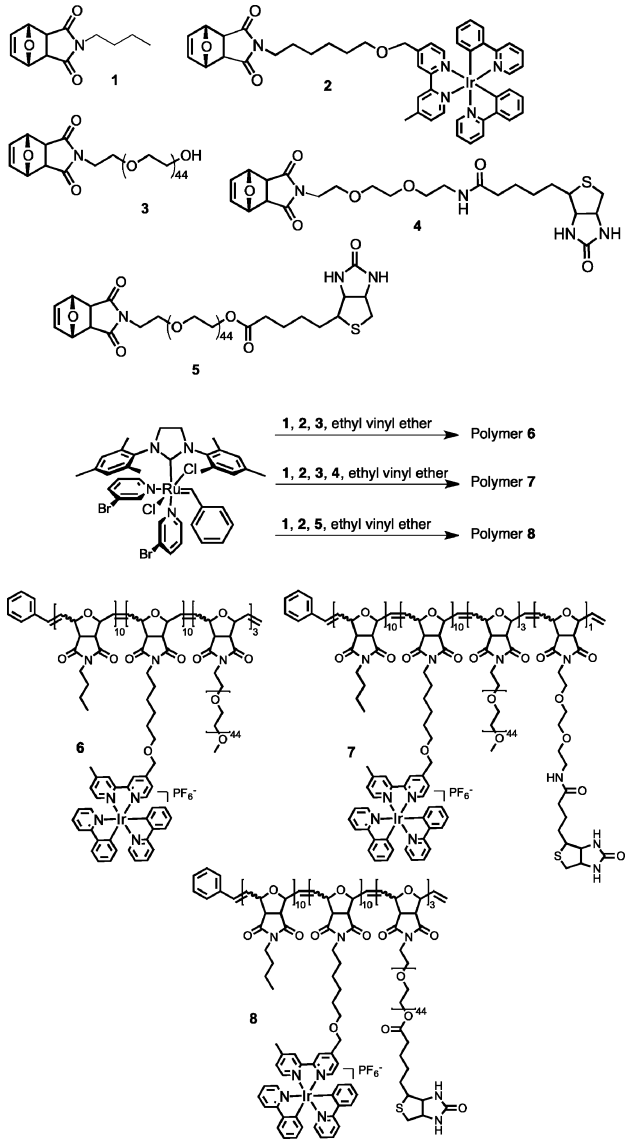
We report here the use of ring-opening metathesis polymerization (ROMP)[5c,10] to generate iridium-containing block copolymers, with the goal of using their self-assembled micelles as luminescent markers in biodetection assays. Interestingly, when these polymers self-assemble into micelles, an enhancement in the iridium quantum yield is observed. In addition, the placement of biotin in the polymer was essential to the streptavidin-binding ability of the final micelle. Micelles composed of polymers simply terminated with biotin monomers did not bind to streptavidin, but when a new biotin-labeled PEG macromonomer was incorporated into the polymer, micelles with exposed biotin were generated that could bind to streptavidin. Binding was ascertained both on streptavidin-coated magnetic particles by fluorescence spectroscopy and on streptavidin micropatterned glass surfaces by fluorescence microscopy. From a broader perspective, this new ROMP macromonomer presents a useful approach to create self-assembled micelles with biorecognition elements displayed on the micelle surface and will increase the application range of ROMP micelles in biosensing, drug delivery, and tissue engineering.

To build iridium containing block copolymers for biodetection, we first synthesized monomers **1** (C4),[10a] *fac*-**2** (Ir), **3** (PEG), and **4** (Biotin).[5c,11] Block copolymers **6** and **7** were synthesized by sequential polymerization of monomers **1**−**3** or **1**−**4**, respectively, using ROMP with the third generation ruthenium-based Grubbs catalyst (Scheme 2). The presence of the iridium complex made GPC characterization difficult, but ^{1}H NMR analysis indicated that the block ratios in the final polymer were consistent with the monomer ratios added during the polymerization. Previous work in our laboratory demonstrated the living nature of these polymerizations.[10b]

In block copolymers **6** and **7** the C4 and luminescent Ir blocks are hydrophobic, while the PEG-based block is water-soluble. Polymer **6** contains no biotin, while in polymer 7, one biotin moiety, on average, is attached at the water-soluble end of the polymer chain via biotin-based ROMP monomer **4** (Scheme 2).

The self-assembly of these polymers was induced by adding water to acetonitrile solutions of the polymers (Supporting Information). The solutions formed upon water addition to polymers **6** and **7** remained clear, and transmission electron microscopy (TEM) results indicated that micelles were predominantly formed with an average diameter of 20 nm, as well as a minor proportion of larger spherical particles (Supporting Information). With a large metal-containing micellar core, the use of macromonomer **3** with long PEG chains was required to ensure that self-assembly resulted in star micelles rather than solely in large compound micelles.[5c] Using this monomer, only a few repeat units were necessary for self-assembly into micelles. By TEM, on dried samples after solvent removal, it is likely that only the metal-containing core is visible and that the PEG corona does not provide sufficient contrast

Scheme 2. Monomers and Polymers



for imaging. Indeed, atomic force microscopy (AFM) imaging on mica surfaces showed that the average height of the micelles of **6** is closer to 50 nm as it includes the PEG corona (Supporting Information).

The quantum yield and emission maxima for monomer **2** (in acetonitrile) and polymers **6** and **7** in their unassembled form (acetonitrile) and as micelles (acetonitrile/water) are shown in Table 1. Measurements in acetonitrile were conducted under argon using thoroughly degassed solvents. For aqueous samples containing micelles, no particular precautions were employed to

Table 1. Quantum Yields of Ir(III) Monomer and Polymers

sample	solvent	Φ	λ_{em}
monomer **2**	MeCN	0.28	579
polymer **6**	MeCN	0.24	581
	90% water	0.40	565
polymer **7**	MeCN	0.24	580
	90% water	0.34	562
polymer **8**	MeCN	0.24	580
	90% water	0.31	563

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protect the samples from air. *fac*-Ir(ppy)$_3$ (ppy = 2-phenylpyridine) was used as the standard for the quantum yield measurements (Φ = 0.4).[8e,12]

There are two main differences between the emission spectra of the open polymer chains in acetonitrile and the micelles in aqueous solution. First, there is a blue shift of approximately 15 nm upon micellization in water (Table 1, Figure 1). This blue

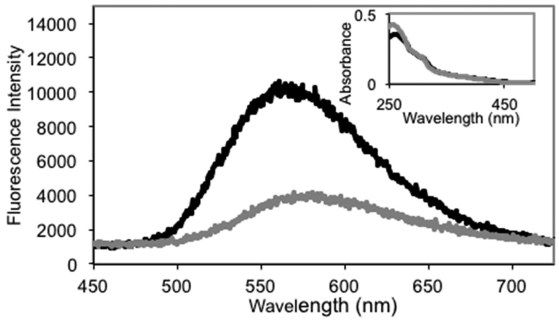

Figure 1. Fluorescence spectra of **6** as open polymer chains in degassed acetonitrile (gray) and as micelles in water (black). Inset: the absorbance spectra of the same samples, showing similar absorbance at the excitation wavelength of 370 nm.

shift could be a result of desolvation of the core and/or core rigidity, as the iridium complexes are confined within the micelle core.[13] The excited state of Ir(ppy)$_2$(bpy) complexes is at least partially ^{3}MCLT (metal to ligand charge transfer) in character.[14] When these complexes are excited in fluid solution, solvent molecules can reorient to stabilize this excited state, and emission occurs from this relaxed state. When the complex is excited in a rigid environment, however, solvent reorientation is reduced on the time scale of emission, and a blue shift may be observed.

The second observation is that the quantum yield of these unassembled polymer chains in acetonitrile is similar to that of monomer **2** and the values reported for related *fac*-Ir(ppy)$_2$(bpy) PF_6 complexes,[8a,15] indicating no self-quenching as a result of incorporation into a polymer. Interestingly, the quantum yield *increases* in water upon micellization (Table 1, Figure 1). This quantum yield increase was surprising, since the samples were exposed to air and water, which may be expected to quench the luminescence. There are literature reports of increased iridium luminescence attributed to increased hydrophobicity and rigidity of the local environment. For example, monomeric iridium complexes that are conjugated to biotin molecules demonstrate an increased quantum yield upon binding to streptavidin,[16] and iridium complexes have also shown luminescence enhancement upon binding (through ligand-binding interactions) to BSA (bovine serum albumin) and DNA.[17] In other examples, some iridium complexes with specific structures were shown to have higher quantum yields in the solid state than in solution. The exact mechanism for this observed increase has been debated but may be due to aggregation of the complexes, resulting in excited state interactions between ligands of closely packed complexes or to restricted motion of the complexes.[18−21] In the case of our polymers, the iridium complexes, as well as the butyl groups that comprise one block of the polymer, become aggregated into a micelle core having a diameter of approximately 20 nm. Water is excluded from this core, and acetonitrile also likely diffuses out due to the gradient of acetonitrile between the inner core and the outer solution. The core is thus a densely packed, hydrophobic environment, and this may account for our observed increased quantum yield for the iridium complexes in these micelles.

These micelles contain a large number of iridium centers, and their luminescence is not just additive, but enhanced, making them bright probes that can significantly increase the sensitivity of bioassays. To measure the binding affinity of our micelles to streptavidin, we used streptavidin-coated magnetic beads.[22]

In this experiment, a micelle sample (of polymer **7**) was added to both a reaction solution containing a suspension of the magnetic beads, and to a control solution of the same buffer as the reaction solution, but containing no beads. After incubation, the reaction solution was separated from the beads. The fluorescence of this reaction solution was compared to the control solution. Any decrease in the fluorescence of the reaction solution compared to the control would be due to the removal of luminescent micelles from the solution by biotin−streptavidin (bead) interactions.

For this experiment to be successful, it was necessary to suppress nonspecific binding interactions and retain specific biotin-streptavidin binding. A number of reaction and washing buffers were explored, using nonbiotinylated polymer **6** and a monomeric iridium-biotin molecule as controls. Our best results were obtained when PBS buffer containing 0.05% Tween 20 was used for prewashing the streptavidin-coated magnetic beads and for the micelle-bead conjugation reaction. After the conjugation reaction and the isolation of the reaction solution, the beads were washed with an aqueous solution of 0.1% Tween 20 to remove any micelles bound to the beads by nonspecific interactions (micelles are stable in this solution).

The incubation of biotinylated polymer **7** with streptavidin magnetic beads yielded a solution having very similar emission as the control solution containing no beads, indicating that this polymer did not bind significantly to the streptavidin on the beads (Figure 2B).

Previous reports have demonstrated that biotinylated micellar structures can bind to streptavidin,[23−27] although in cases where the biotin exposure on the surface is quantified, most results show that only a fraction (<25%) of the biotin units are actually available for binding.[28−30] Polymer **7** has, on average, a single biotin unit on the end of each polymer chain. The biotin is likely completely surrounded by the neighboring PEG chains and, based on these results, inaccessible to streptavidin. The subsequent washes with 0.1% Tween 20 (solid gray line) indicated, although qualitatively only, that essentially no material was bound nonspecifically.

In an attempt to improve micelle binding to streptavidin, we synthesized the novel macromonomer **5** (Supporting Information), in which a long PEG chain was functionalized at one end with the norbornene-based ROMP monomer and at the other end with a biotin moiety (Scheme 2).

Macromonomer **5** was incorporated into the new triblock copolymer **8** (Scheme 2). Self-assembly experiments with this polymer were conducted in the same manner as polymers **6** and **7**. When water was added to acetonitrile solutions of polymer **8**, the solutions became slightly cloudy at approximately 50% water, which is possibly consistent with the presence of the somewhat hydrophobic biotin groups on the micelle surface. TEM analysis showed that micelles were still predominantly formed with a core size of 29 nm on average, along with some larger spherical aggregates. AFM height

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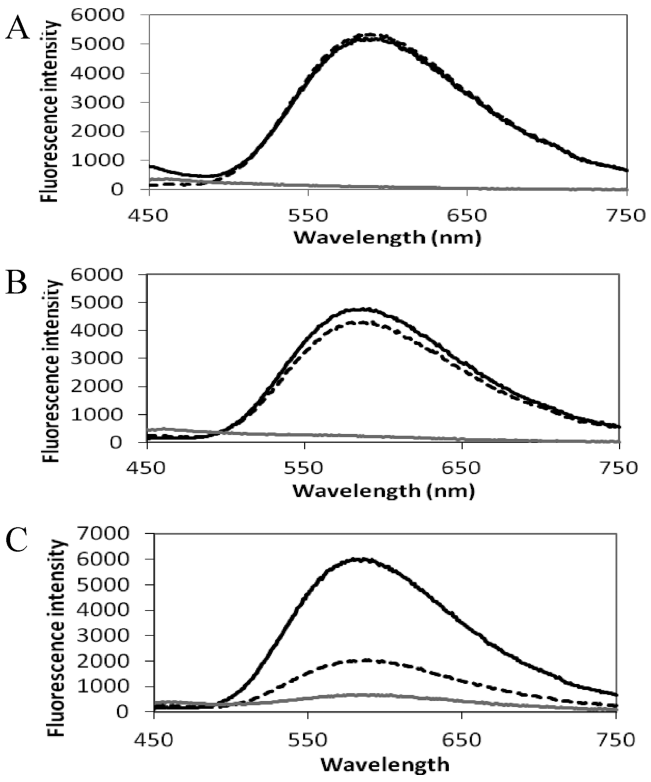

Figure 2. Fluorescence spectra of biotin–streptavidin conjugation reaction solutions. A: polymer **6**; B: polymer **7**; C: polymer **8**. Solid black curve: control solution (no beads); dotted black curve: reaction solution (from polymer + beads); gray curve: 0.1% Tween 20 wash.

analysis confirmed that the micelles are slightly larger than those of **6**.

In the micelles from **8**, the biotin units are at the end of the long PEG chains. We hoped that this would increase the likelihood of biotin units being displayed on the micelle surface. Indeed, in our magnetic bead experiments, we observed a significant decrease in luminescence of the reaction solution compared to the control solution containing no beads (Figure 2C), indicating that this polymer was capable of binding to the beads. That only very minimal nonspecific binding occurred was confirmed by measuring the emission of the 0.1% Tween 20 washes (Figure 2C, gray line). Semiquantitative evaluation of this binding affinity showed that, on average, 1 mg of steptavidin-coated beads was able to bind approximately 1.2 nmol of polymer (Supporting Information). This estimate is on the same order of magnitude as binding of biotinylated IgG to these beads (0.5 nmol per mg of beads).[31] These magnetic bead tests confirmed that the redesigned polymer **8** micelles indeed have biotin displayed on the micelle surface and that the micelles are able to recognize and bind to biomolecule targets.

To further explore their potential as luminescence probes in bioassays, we examined the ability of these micelles to bind to specific targets on surfaces. In these preliminary experiments, a poly(dimethyl siloxane) stamp patterned with lines was inked with a solution of streptavidin labeled with the fluorescent dye Dylight 405, and the streptavidin was microcontact-printed onto a functionalized glass coverslip (Supporting Information). Next, a pegylated blocking agent (poly(lysine)-graft-PEG) was applied to the coverslips to minimize nonspecific binding, followed by incubation with a droplet of solution containing micelles **8** and rinsing (Scheme S1, Supporting Information). The use of stamped patterns allowed differentiation between binding to streptavidin and nonspecific interactions with the glass surface.

The microcontact-printed pattern and the micelles were imaged by fluorescence microscopy using two different fluorescence imaging channels. Figure 3 shows an image

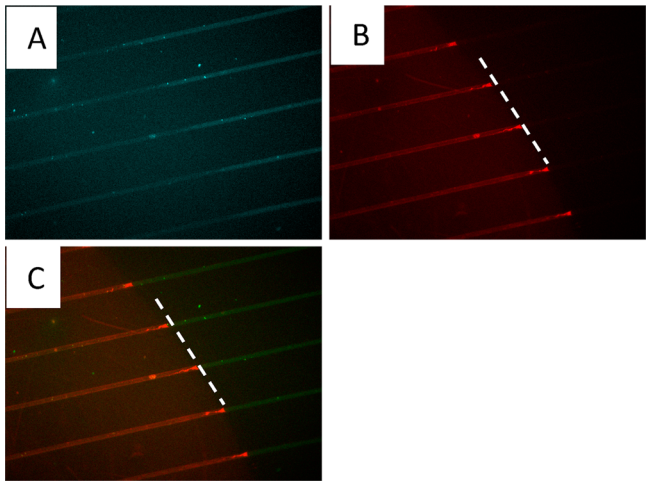

Figure 3. Fluorescence microscopy images of STV and STV incubated with micelles of **8**. A: printed streptavidin-Dylight 405; B: micelle emission; C: colocalized emission image. The dotted white line indicates part of the edge of the micelle droplet. The stripes are 10 μm wide and 110 μm apart.

captured at the edge of the droplet and shows the printed stripes of streptavidin-Dylight 405 (Figure 3A) and the fluorescence emission of the micelles (Figure 3B). The edge of the micelle droplet is partially marked with a dashed line. Figure 3C displays the digital superposition of both images and confirms that the micelles of polymer **8** specifically bind to the streptavidin patterned onto the glass surface. Interestingly, the fluorescence pattern of the micelles, but not the printed pattern of the streptavidin, is enhanced close to the edge of the droplet. This may be accounted for by the "coffee staining" effect, where a concentrated solute ring is left around the edge of a drop upon evaporation.[32] Note that the fluorescence enhancement is confined to the stripes, further suggesting that binding of micelles **8** to the surface is driven by specific biotin–streptavidin interactions. These results indicate that it is indeed possible to use the micelles as fluorescent labels for solid phase immunoassays by using antibodies conjugated with biotin.

In conclusion, we have designed luminescent Ir(III)-containing block copolymers that exhibit an enhancement in quantum yield upon micellization. Biotin moieties were included in the polymers through the synthesis and incorporation of biotin-containing ROMP monomers. The polymer design was a very important factor in determining the biotin availability on the micelle surface and the ability of the micelle to bind to streptavidin. Attaching a biotin monomer at the end of the ROMP polymer was not sufficient to allow for micelle–streptavidin binding because the small biotin moiety was likely shielded by the PEG portion of the polymer chain. A new macromonomer was designed that allowed for the biotin functionalities to be displayed on the micelle surface, resulting in greatly improved micelle–streptavidin binding. The new biotin-functionalized micelles were able to bind to streptavidin-coated magnetic beads suspended in solution as well as

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streptavidin printed onto glass surfaces, making the polymers useful candidates as probes in biodetection assays. We are currently optimizing the binding of the micelles to modified glass surfaces, investigating the binding of these micelles in flow devices and in microfluidic arrays and exploring their potential in electrochemiluminescence designs.

■ ASSOCIATED CONTENT

Supporting Information

Materials, reagents, instruments, and detailed experimental methods; synthetic procedures and characterization for monomer **5** and polymers **6**–**8**; calculation of binding of **8** to magnetic beads; complete details of the coverslip preparation, microcontact-printing, incubation, and microscopy. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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(22) To determine the binding affinity of biotin-labelled polymers **7** and **8**, we attempted to use the HABA displacement assay. In this commonly used assay, the small molecule HABA (2-(4′-hydroxyphenylazo)benzoic acid) is added to streptavidin and shows a characteristic absorbance at 500 nm upon binding. When a biotinylated sample is added to the HABA–streptavidin solution, the strong biotin–

streptavidin binding causes the expulsion of the more weakly bound HABA molecules into the aqueous medium, and a decrease in the absorbance at 500 nm is observed. Unfortunately, for these polymer samples the iridium complexes incorporated into the polymers demonstrated a small but noticeable absorbance at 500 nm which interfered with the assay.

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