THE JOURNAL OF PHYSICAL CHEMISTRY Letters

Letter

pubs.acs.org/JPCL

Molecular Weight Determination by Counting Molecules

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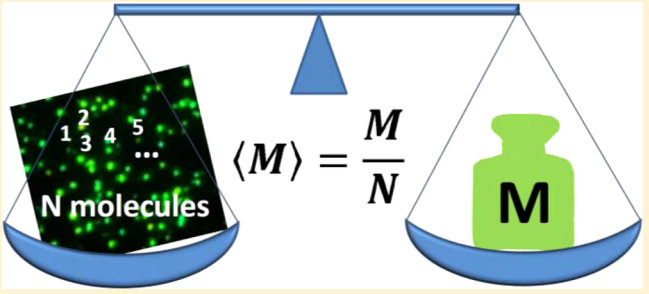
Supporting Information

ABSTRACT: Molecular weight (MW) is one of the most important characteristics of macromolecules. Sometimes, MW cannot be measured correctly by conventional methods like gel permeation chromatography (GPC) due to, for example, aggregation. We propose using single-molecule spectroscopy to measure the average MW simply by counting individual fluorescent molecules embedded in a thin matrix film at known mass concentration. We tested the method on dye molecules, a labeled protein, and the conjugated polymer MEH-PPV. We showed that GPC with polystyrene calibration overestimates the MW of large MEH-PPV molecules by 40 times due to chain aggregation and stiffness. This is a crucial observation for understanding correlations between the conjugated polymer length, photophysics and performances of devices. The method can measure the MW of fluorescent molecules, biological objects, and nanoparticles at ultimately low concentrations and does not need any reference; it is conformation-independent and has no limitations regarding the detected MW range.



Properties of polymers and other macromolecules are crucially dependent on their molecular weight (MW).[1] MW can be measured using many different methods including gel permeation chromatography (GPC), osmometry, light scattering, viscometry, cryoscopy, ebulliometry, ultracentrifugation, mass spectrometry, and end-group analysis.[2−6] However, these methods typically require a high sample concentration, which can cause unreliable results for analytes with aggregation tendencies such as conjugated polymers.

Among all, GPC is the most popular method,[7,8] which is relatively simple and able to handle nonpurified samples and measure MW distribution over a broad working range. GPC measures the retention time that macromolecules experience as they pass through a column packed with porous beads. This time depends on the size and shape of the molecule. To convert the time to the MW, calibration against a polymer with similar shape, small polydispersity index (PDI), and known MW is needed. However, the calibration is often not straightforward. For example, GPC overestimates the MW of semiflexible conjugated polymers if, according to standard practice, a flexible polymer such as polystyrene (PS) is used for relative calibration.[9−12] Moreover, the flexibility of conjugated polymers depends on a tiny (usually unknown) concentration of chemical defects. Universal calibration[13] can provide the absolute MW but requires detection of the viscosity, for which a high concentration is necessary, giving results susceptible to analyte aggregation.

Single-molecule spectroscopy (SMS)[14,15] allows direct investigation of individual molecules without ensemble averaging.[16−18] It provides the opportunity to count the molecules by counting the individual diffraction-limited spots in the fluorescence images. The number of spots is proportional to the sample concentration.[19,20] Although such counting is a routine practice in the SMS field, it is rarely employed for any quantitative analysis.[21,22] In this work, we demonstrate that quantitative molecule counting can be used to determine the MW of fluorescent macromolecules. In order to exemplify some of the limitations that are associated with GPC but not the technique proposed here, we chose to work with the conjugated polymer poly[2-methoxy-5-(2-ethylhexyloxy)-1,4-phenylenevinylene] (MEH-PPV), which is known to aggregate in organic solvents[23] even at concentrations as low as 1 nM.[24]

A typical sample for SMS is prepared by dispersion of analyte molecules in a matrix thin film at ultralow concentration using spin-casting.[17,25] If we know the concentration (g/L) of the analyte and the matrix polymer in the spin-casting solution and assuming that their relative concentration is the same in the solution and in the dried matrix film, we can use the mass of the matrix polymer as the reference to calculate the total mass of the analyte molecules in the film (m_{analyte}) within the excited area (S)

$$m_{\mathrm{analyte}} = \frac{C_{\mathrm{analyte}} \rho_{\mathrm{matrix}} d_{\mathrm{film}} S}{C_{\mathrm{matrix}}} \quad (1)$$

Received: February 10, 2015
Accepted: February 23, 2015
Published: February 23, 2015

DOI: 10.1021/acs.jpclett.5b00296
J. Phys. Chem. Lett. 2015, 6, 923−927

where $C_{analyte}$ and C_{matrix} are the concentrations (g/L) of the analyte and the matrix in the spin-casting solution, ρ_{matrix} is the matrix density (e.g., 1.18 g/cm^3 for poly(methyl methacrylate) (PMMA) and 1.25 g/cm^3 for poly(vinyl alcohol) (PVA)), and d_{film} is the thickness of the matrix film. The thickness can be measured by AFM, ellipsometry, or profilometry; see the Supporting Information (SI).

From the fluorescence images (Figure S1 in the SI), we count the number of bright spots, which corresponds to the number of the individual analyte molecules (N). The number-average MW (M_n) can be calculated according to its definition

$$M_n = \frac{\sum M_i N_i}{\sum N_i} N_A = \frac{m_{analyte}}{N} N_A \quad (2)$$

where N_A is Avogadro's constant.

Can the counting be accurate? Limited literature on quantitative SMS analysis indicates that this can be difficult.[21,22] The main reason is the disturbance caused by luminescent impurities, which is a problem for SMS in general. It is well established that it is unfeasible to completely avoid luminescent impurities especially for blue-light excitation. However, one can take into account the contribution from impurities by counting molecules in samples with different concentrations of the analyte as well as in a blank sample.

To test the single-molecule counting (SMC) method, we measured the MW of the dye *N*,*N*′-bis(2,6-dimethylphenyl)-perylene-3,4,9,10-tetra-carboxylicdiimide (DXP, Sigma-Aldrich) and large rabbit antimouse immunoglobulin G (IgG) protein molecules labeled with Alexa Fluor 633 (Life Technologies). PMMA and PVA were used as matrices for DXP and IgG, respectively. To decrease the concentration of impurities, PMMA was precleaned by precipitation as reported.[26] We counted the number of fluorescence spots per unit area (spot density $n = N/S$) for several samples with different concentrations of DXP and IgG. Considering that impurities give spot density of $n_{imp} = N_{imp}/S$ and combining eqs 1 and 2, we obtain

$$n_{total} - n_{imp} = \frac{C_{analyte}\rho_{matrix} d_{film} N_A}{C_{matrix} M_n} \quad (3)$$

Hence, the MW is inversely proportional to the slope of $n_{total}(C_{analyte})$ on the linear scale. By taking the logarithm of both sides, we can express the spot density as a function of $C_{analyte}$ in log–log scale

$$\log(n_{total} - n_{imp}) = \log\left(\frac{\rho_{matrix} d_{film} N_A}{C_{matrix}}\right) + \log(C_{analyte}) - \log(M_n) \quad (4)$$

where n is in cm^{-2}, C is in g/L, N_A is in mol^{-1}, ρ is in g/cm^3, d is in cm, and M_n is in g/mol. Therefore, the logarithm of the MW is proportional to the horizontal shift of the linear fits of the experimental data along the x-axis in log–log scale. The linear relationship between the spot density and the concentration shown in Figure 1 indicates no substantial aggregation or overlapping of diffraction-limited spots belonging to different molecules.

As shown in Figure S2 in the SI, the intersections of the linear fits with the solid vertical lines (zero concentration) are in good agreement with the spot densities experimentally measured for blank samples. Therefore, the slope obtained from the linear fit accurately represents the relationship between the analyte spot density and the concentration.

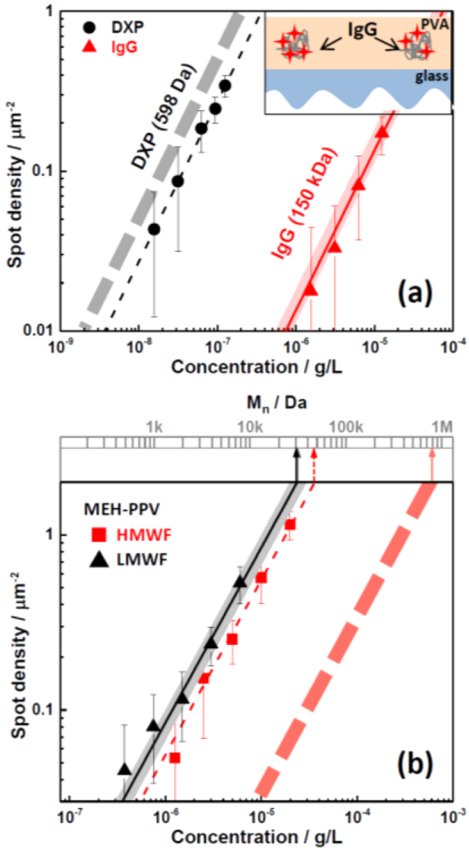


Figure 1. Fluorescence spot densities as a function of the analyte concentration in the spin-casting solutions of the analytes: (a) the DXP dye (dashed lines) and protein IgG (solide lines); (b) two GPC fractions of MEH-PPV (HMWF by dashed lines and LMWF by solid lines). Each data point was obtained by averaging 20 images with the error bar showing the standard deviation. Thin lines are the linear fits to the data. Broad lines are the expected dependencies obtained from eq 4 using the MWs known either from the structure (DXP, IgG) or from GPC (MEH-PPV). The logarithm of the MW is proportional to the horizontal shift of the lines. For (b), the MW can be directly read from the scale bar on the top, as indicated by the arrows. The inset shows the structure of the samples.

As shown in Figure 1a, for the DXP, we experimentally observed about 40% fewer molecules than expected according to the known MW of DXP (M_n = 599 Da). Therefore, M_n of DXP measured by SMC becomes 970 Da, which is an overestimation. The reason for this mismatch could be photobleaching, quenching, or the orientation of the dye (see the SI for discussion). Experiments are ongoing to understand the origin of this discrepancy. For the IgG protein, MWs of 160 kDa were obtained, which is in excellent agreement with the expected value (150 kDa). One of the reasons for the much higher accuracy obtained for IgG could be the presence of several Alexa Fluor 633 dyes per protein. This not only leads to a higher brightness but also detects the molecule even if some of the labels are bleached. Moreover, fluorescence excitation at 633 nm makes contribution from impurities almost negligible. We would like to stress that the SMC method is developed for large molecules. For small molecules, the MW can be obtained by other precise means or methods. In general, we can conclude that these test experiments successfully demonstrate

DOI: 10.1021/acs.jpclett.5b00296
J. Phys. Chem. Lett. 2015, 6, 923–927

the principle of the SMC method and that the data are in reasonable agreement with the theoretically expected values.

The SMC experiment was performed for the conjugated polymer MEH-PPV (Sigma-Aldrich) dispersed in PMMA. Because the commercial polymer has a large PDI, we fractionated the polymer by GPC and collected a high MW fraction (HMWF) with M_n = 2 MDa, PDI = 1.11 and a low MW fraction (LMWF) with M_n = 80 kDa, PDI = 1.18 relative to PS (Figure 2). MEH-PPV and similar conjugated polymers

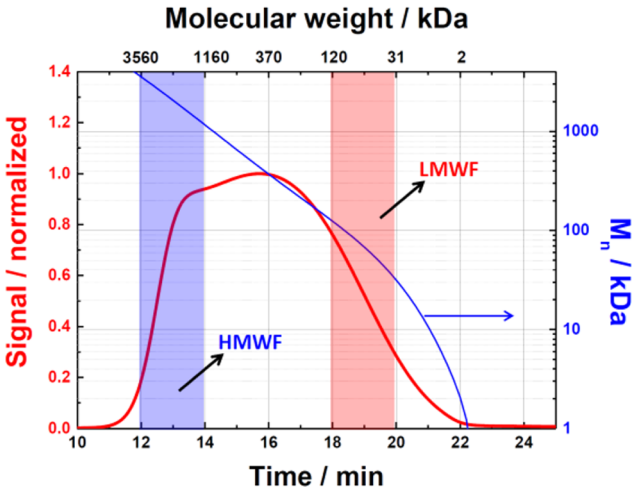


Figure 2. Chromatography of a MEH-PPV stock solution (0.1 g/L, thick line). The HMWF and LMWF were collected at 12−14 and 18−20 min (marked regions), respectively. The M_n/elution time calibration curve obtained using PS standards is shown by a thin line.

are semiflexible. The persistence length is ∼6 nm,[27] which is 6 times larger than that of PS (flexible reference polymer). Because of this, GPC with PS calibration overestimates the MW by a factor of 2−3 for semiflexible conjugated polymers.[9,10] Thus, we divided the MW obtained by GPC with PS calibration by 2.5[28] (correction factor for stiffness) to obtain more realistic values of the apparent MW of the MEH-PPV fractions, $M_n \approx$ 800 kDa for HMWF and $M_n \approx$ 32 kDa for LMWF.

Figure 1b shows that the M_n obtained by SMC for LMWF is 31 kDa, which is in a good agreement with the GPC data (corrected for stiffness), while a very large discrepancy was observed for HMWF. We counted in HMWF about 16 times (40 times without correction for stiffness) more molecules than expected. In terms of MW, it means 47 kDa by SMC as compared to 800 kDa by GPC (corrected for stiffness). The mismatch must be caused by limitations of the GPC methodology because by removing the contribution from impurities, we have taken into account all likely reasons for counting too many spots by the SMC method.

To further understand the origin of this large discrepancy, we analyzed HMWF by GPC under several different conditions. Because the concentrations were very low, fluorescence detection was used. As shown in Figure 3, a shoulder and a peak at ∼22 min in the low MW range clearly increase with decreasing concentration (from 3×10^{-3} to 2×10^{-5} g/L, corresponding to an optical density from 0.2 to 1.5×10^{-3} for a 1 cm path length at 514 nm). The main peak at around 12−15 min also shifts slightly toward lower MW upon dilution. In addition, after keeping the solution of the lowest concentration at 50 °C in the dark for two days, a further enhancement of the low MW peak is observed. Such concentration and temperature

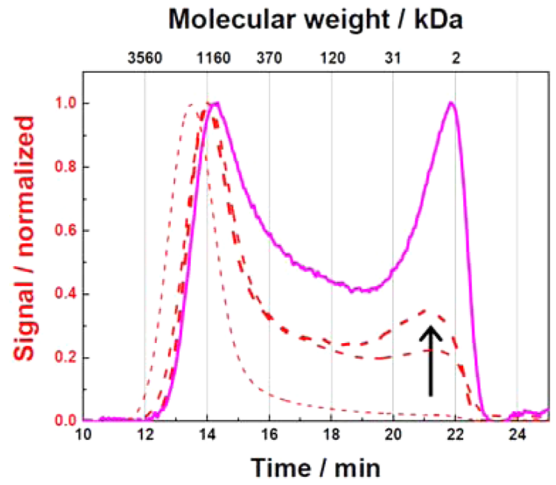


Figure 3. Rechromatography of the HMWF at different concentrations (3×10^{-3}, 1×10^{-4}, and 2×10^{-5} g/L, dashed lines; the arrow indicates the decreasing concentration) and after keeping the 2×10^{-5} g/L solution in the dark at 50 °C for 2 days (solid thick line). Due to the capacity of the column, the resolution was poor for MW < 20 kDa. Thus, the peak at 22 min represents all molecules that are lighter than 20 kDa relative to PS. Fluorescence spectra of all solutions were identical (Figure S1 in the SI).

dependences are typical for a disaggregation process. We cannot completely exclude chain cleavage of MEH-PPV during the heating treatment. However, 20 times decrease of the MW in the dark is very unlikely because the chain cleavage of MEH-PPV is photoinduced.[29] Moreover, when the same experiments were done for MEH-PPV solutions at higher concentration, no changes of the GPC traces were observed, suggesting that the low MW part comes from disaggregation rather than degradation. Finally, as discussed in detail in the SI, the peak at ∼22 min cannot be caused by the impurities.

Evidently, if molecules are aggregated, GPC or, in fact, any other technique gives masses (and mass distributions) of the aggregates but not of the individual molecules. This is the principal reason for the considerable discrepancy between the M_n obtained by SMC at ultralow concentration when the aggregates are mostly dissociated (the spin-casting solution had a concentration of $\sim 10^{-6}$ g/L) and the much higher M_n measured by GPC at standard concentrations. Thus, GPC data for conjugated polymers like MEH-PPV can be used for quantitative comparisons only if the concentration of the analyte is very low (10^{-5} g/L or less), which totally excludes detection by viscosity or absorption. Only fluorescence-based SMC and possibly fluorescence-detection GPC are suitable for studying samples at concentrations close to the aggregation-free condition.

The strong aggregation tendency of conjugated polymers at moderate and high concentrations (>0.1 g/L) has been known for years.[23] At the same time, the majority of published research that correlates the photophysics of conjugated polymers with their MW does not take into account the aggregation effect when concentrated solutions were used for GPC fractionation,[30−33] and the results of the MW determination were never doubted. The same problem concerns conjugated polymers provided by commercial suppliers where GPC (measured at unknown high concentration using PS calibration) serves as the only indication of the product MW.

DOI: 10.1021/acs.jpclett.5b00296
J. Phys. Chem. Lett. 2015, 6, 923−927

Our results confirm recent data of Wang et al., which suggests that MEH-PPV aggregates can be stable even at quite low concentrations of 1 nM.[24] The good agreement between SMC and GPC for the low MW fraction of MEH-PPV indicates that the aggregation tendency for small MEH-PPV molecules is much weaker, which is also in agreement with the conclusion by Wang et al.[24] Note, however, that even in that study, GPC at concentrations orders of magnitude higher than 1 nM was used for the MW separation.[24]

The accuracy of SMC depends on the accuracy of the determination of the analyte mass and the detection efficiency of the molecules. For single chromophoric molecules (like the DXP dye used here), the detection efficiency gives probably the major error due to the low fluorescence signal, photobleaching, and/or permanent quenching. For multichromophoric systems (like conjugated polymers or fluorescence-labeled proteins), the detection is not a problem. Then, the error mainly comes from film thickness variations, which in our case is up to ±20%. To minimize these variations, we performed both profilometry and AFM to get reliable values. In addition, it is worth noting that the film thickness was measured for the very same films as those used for the SMC measurements. Because the thickness measurements give absolute errors, using thicker films is preferable (see the SI for more discussion about the accuracy).

In conclusion, we have shown that quantitative SMC can be successfully applied for average MW determination of complex systems at conditions when other techniques like GPC are prone to errors (e.g., when molecules/particles have strong aggregation or by any other reasons working at nanomolar concentrations is required). We showed that GPC with PS calibration can overestimate the MW of the conjugated polymer MEH-PPV due to the chain conformation and aggregation in solution. Note that by default, commercial suppliers characterize their conjugated polymers by GPC with PS calibrations, which may overestimate the MW especially for batches with the declared MW larger than 100 kDa. In addition, SMC is reference-free, conformation-independent (because molecules are counted regardless of their shape and size), and can access an unlimited MW range. SMC can also be applied to nonfluorescent macromolecules if they are labeled with fluorescent markers, which is a standard approach in biological imaging. It is important to note that SMC does not rely on fluorescence brightness. Therefore, it does not matter how the molecules are labeled. In general, labeling with many dyes per molecule (as a usual case for, e.g., fluorescent proteins) is preferred.

We stress that our report introduces the principle of SMC that should be further developed to optimize the method and find optimal conditions. For example, instead of relying on the polymer matrix thickness measurement as the indicator of the analyte mass, one can use extra reference fluorescent molecules (or fluorescent beads) of known MW and concentration, which can be spectrally distinguished from the analyte molecules. Then, counting them separately, one would be able to determine the MW of the analyte. Moreover, the approach of counting molecules in a known volume can probably be realized in solution phase by means of, for example, fluorescence correlation spectroscopy.

■ ASSOCIATED CONTENT

Supporting Information

Sample preparation; thickness measurement; single-molecule counting; errors in detection of molecules; effect from impurities; inhomogeneity of the sample; compatibility between the dopant and matrix; fluorescence spectra of MEH-PPV in solution; and estimation of the accuracy. 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.

■ ACKNOWLEDGMENTS

This study was financially supported by The Swedish Research Council, the Knut & Alice Wallenberg Foundation, the Crafoord Foundation, as well as the Chalmers Areas of Advance Energy, Materials Science and Nanoscience and Nanotechnology. A.D.Z.M. is supported by the Linnaeus Centre for Bioinspired Supramolecular Function and Design (SUPRA). J.H. and M.W. thank the Erasmus programme for internship funding. We are grateful to Dr. Margareta Sandahl (Centre for Analysis and Synthesis, Lund University) for the possibility to use the fluorescence detection GPC equipment.

■ REFERENCES

(1) Flory, P. J. *Principles of Polymer Chemistry*, 1st ed.; Cornell University Press: Ithaca, NY, 1953.

(2) Flory, P. J. Molecular Weights and Intrinsic Viscosities of Polyisobutylenes. *J. Am. Chem. Soc.* **1943**, *65*, 372−382.

(3) Glover Clyde, A. In *Polymer Molecular Weight Methods*; Ezrin, M., Ed.; Advances in Chemistry; American Chemical Society: Washington, DC, 1973; Vol. *125*, pp 1−8.

(4) Mikulak, R.; Runquist, O. Molecular Weights by Cryoscopy: A General Chemistry Laboratory Experiment. *J. Chem. Educ.* **1961**, *38*, 557.

(5) Li, W.; Chung, H.; Daeffler, C.; Johnson, J. A.; Grubbs, R. H. Application of ^{1}H DOSY for Facile Measurement of Polymer Molecular Weights. *Macromolecules* **2012**, *45*, 9595−9603.

(6) Hanton, S. D. Mass Spectrometry of Polymers and Polymer Surfaces. *Chem. Rev.* **2001**, *101*, 527−570.

(7) Moore, J. C. Gel Permeation Chromatography. I. A New Method for Molecular Weight Distribution of High Polymers. *J. Polym. Sci., Part A: Gen. Pap.* **1964**, *2*, 835−843.

(8) Lathe, G. H.; Ruthven, C. R. The Separation of Substances and Estimation of Their Relative Molecular Sizes by the Use of Colums of Starch in Water. *Biochem. J.* **1956**, *62*, 665−674.

(9) Grell, M.; Bradley, D. D. C.; Long, X.; Chamberlain, T.; Inbasekaran, M.; Woo, E. P.; Soliman, M. Chain Geometry, Solution Aggregation and Enhanced Dichroism in the Liquidcrystalline Conjugated Polymer Poly(9,9-dioctylfluorene). *Acta Polym.* **1998**, *49*, 439−444.

(10) Becker, K.; Gaefke, G.; Rolffs, J.; Höger, S.; Lupton, J. M. Quantitative Mass Determination of Conjugated Polymers for Single Molecule Conformation Analysis: Enhancing Rigidity with Macrocycles. *Chem. Commun.* **2010**, *46*, 4686−4688.

(11) Liu, J.; Loewe, R. S.; McCullough, R. D. Employing MALDI-MS on Poly(alkylthiophenes): Analysis of Molecular Weights, Molecular Weight Distributions, End-Group Structures, and End-Group Modifications. *Macromolecules* **1999**, *32*, 5777−5785.

(12) Koch, F. P. V; Smith, P.; Heeney, M. "Fibonacci's Route" to Regioregular Oligo(3-hexylthiophene)s. *J. Am. Chem. Soc.* **2013**, *135*, 13695−13698.

(13) Grubisic, Z.; Rempp, P.; Benoit, H. A Universal Calibration for Gel Permeation Chromatography. *J. Polym. Sci., Part B: Polym. Lett.* **1967**, *5*, 753−759.

DOI: 10.1021/acs.jpclett.5b00296
J. Phys. Chem. Lett. 2015, 6, 923−927

(14) Moerner, W.; Kador, L. Optical Detection and Spectroscopy of Single Molecules in a Solid. *Phys. Rev. Lett.* **1989**, *62*, 2535−2538.
(15) Orrit, M.; Bernard, J. Single Pentacene Molecules Detected by Fluorescence Excitation in a p-Terphenyl Crystal. *Phys. Rev. Lett.* **1990**, *65*, 2716−2719.
(16) Moerner, W. E.; Fromm, D. P. Methods of Single-Molecule Fluorescence Spectroscopy and Microscopy. *Rev. Sci. Instrum.* **2003**, *74*, 3597.
(17) Moerner, W. E.; Orrit, M. Illuminating Single Molecules in Condensed Matter. *Science.* **1999**, *283*, 1670−1676.
(18) Vanden Bout, D. A.; Yip, W.-T.; Hu, D.; Fu, D.; Swager, T. M.; Barbara, P. F. Discrete Intensity Jumps and Intramolecular Electronic Energy Transfer in the Spectroscopy of Single Conjugated Polymer Molecules. *Science.* **1997**, *277*, 1074−1077.
(19) Tian, Y.; Camacho, R.; Thomsson, D.; Reus, M.; Holzwarth, A. R.; Scheblykin, I. G. Organization of Bacteriochlorophylls in Individual Chlorosomes from Chlorobaculum Tepidum Studied by 2-Dimensional Polarization Fluorescence Microscopy. *J. Am. Chem. Soc.* **2011**, *133*, 17192−17199.
(20) Müller, J. G.; Lupton, J. M.; Feldmann, J.; Lemmer, U.; Scherf, U. Ultrafast Intramolecular Energy Transfer in Single Conjugated Polymer Chains Probed by Polarized Single Chromophore Spectroscopy. *Appl. Phys. Lett.* **2004**, *84*, 1183.
(21) Gai, H.; Wang, Q.; Ma, Y.; Lin, B. Correlations between Molecular Numbers and Molecular Masses in an Evanescent Field and Their Applications in Probing Molecular Interactions. *Angew. Chem., Int. Ed.* **2005**, *44*, 5107−5110.
(22) Jiang, D.; Wang, L.; Jiang, W. Quantitative Detection of Antibody Based on Single-Molecule Counting by Total Internal Reflection Fluorescence Microscopy with Quantum Dot Labeling. *Anal. Chim. Acta* **2009**, *634*, 83−88.
(23) Nguyen, T.-Q.; Doan, V.; Schwartz, B. J. Conjugated Polymer Aggregates in Solution: Control of Interchain Interactions. *J. Chem. Phys.* **1999**, *110*, 4068.
(24) Wang, D.; Yuan, Y.; Mardiyati, Y.; Bubeck, C.; Koynov, K. From Single Chains to Aggregates, How Conjugated Polymers Behave in Dilute Solutions. *Macromolecules* **2013**, *46*, 6217−6224.
(25) Lupton, J. M. Single-Molecule Spectroscopy for Plastic Electronics: Materials Analysis from the Bottom-Up. *Adv. Mater.* **2010**, *22*, 1689−1721.
(26) Tian, Y.; Sheinin, V.; Kulikova, O.; Mamardashvili, N.; Scheblykin, I. G. Improving Photo-Stability of Conjugated Polymer MEH-PPV Embedded in Solid Matrices by Purification of the Matrix Polymer. *Chem. Phys. Lett.* **2014**, *599*, 142−145.
(27) Gettinger, C. L.; Heeger, A. J.; Drake, J. M.; Pine, D. J. A Photoluminescence Study of Poly(phenylene vinylene) Derivatives: The Effect of Intrinsic Persistence Length. *J. Chem. Phys.* **1994**, *101*, 1673.
(28) Feist, F. A.; Zickler, M. F.; Basché, T. Origin of the Red Sites and Energy Transfer Rates in Single MEH-PPV Chains at Low Temperature. *ChemPhysChem* **2011**, *12*, 1499−1508.
(29) Tian, Y.; Kuzimenkova, M. V.; Xie, M.; Meyer, M. M.; Larsson, P.-O.; Scheblykin, I. G. Watching Two Conjugated Polymer Chains Breaking Each Other When Colliding in Solution. *NPG Asia Mater.* **2014**, *6*, e134.
(30) Lin, H.; Tian, Y.; Zapadka, K.; Persson, G.; Thomsson, D.; Mirzov, O.; Larsson, P.-O.; Widengren, J.; Scheblykin, I. G. Fate of Excitations in Conjugated Polymers: Single-Molecule Spectroscopy Reveals Nonemissive "Dark" Regions in MEH-PPV Individual Chains. *Nano Lett.* **2009**, *9*, 4456−4461.
(31) Habuchi, S.; Onda, S.; Vacha, M. Molecular Weight Dependence of Emission Intensity and Emitting Sites Distribution within Single Conjugated Polymer Molecules. *Phys. Chem. Chem. Phys.* **2011**, *13*, 1743−1753.
(32) Bahtiar, A.; Koynov, K.; Ahn, T.; Bubeck, C. Molecular Weight Effects in the Third-Harmonic Generation Spectroscopy of Thin Films of the Conjugated Polymer MEH-PPV. *J. Phys. Chem. B* **2008**, *112*, 3605−3610.
(33) Sun, W.-Y.; Yang, S.-C.; White, J. D.; Hsu, J.-H.; Peng, K.-Y.; Chen, S. A.; Fann, W. Conformation and Energy Transfer in a Single Luminescent Conjugated Polymer. *Macromolecules* **2005**, *38*, 2966−2973.

DOI: 10.1021/acs.jpclett.5b00296
J. Phys. Chem. Lett. 2015, 6, 923−927