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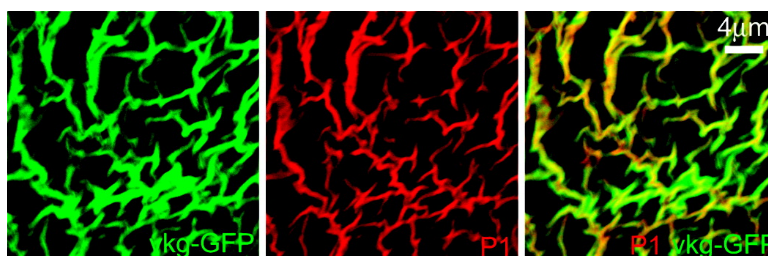

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A Fluorescent Core−Shell Dendritic Macromolecule Specifically Stains The Extracellular Matrix

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In organic solvents, the strongly fluorescent perylene-3,4,9,10-tetracarboxdiimide (PDI) chromophore has been widely applied as a dye in various fields.[1] Recently, water-soluble PDI derivatives[2a−e] and other fluorescent core−shell nanoparticles[2f−i] with biocompatibility have been reported and tested in vivo or in vitro. Among them, a negatively charged PDI core−shell macromolecule bearing multiple carboxylic acid groups has been employed successfully as a fluorescent dye for staining the cell nucleus due to its binding to positively charged histones.[2d] In this communication, we hypothesized that a positively charged macromolecule bearing multiple amines (**P1**, Figure 1A, and S-Scheme 1 and S-Figures 1−3 in Supporting Information) could bind to the highly negatively charged extracellular matrix (ECM) (S-Figure 4) at physiological pH and thus might be applicable to label the ECM of animal tissue. The ECM surrounds cells and plays important roles in many aspects of cellular fate, including cell migration, stem cell differentiation, and cancer progression. Currently, components of the ECM are visualized with low specificity and sensitivity by histochemical means or with much higher detection quality by immunofluorescence using antibodies against individual antigens (Supporting Information). So far, there is no fluorescent dye to directly visualize the ECM network at high resolution. A general fluorescent dye for ECM would, therefore, be useful for many purposes. Herein, a positively charged fluorescent core−shell dendritic macromolecule **P1** was employed for staining animal tissue.

P1 is composed of a central PDI chromophore, a rigid first-generation polyphenylene dendrimer scaffold for suppressing aggregation of the central PDI chromophore,[2c] and a flexible polymer shell with eight arms bearing multiple monofunctional amine groups for inducing water solubility, positive charges, and biological specificity. **P1** showed good water solubility (>10 g/L), thus facilitating its study in a biological environment. A hydrodynamic radius of 4.2 nm was found in water for **P1** by fluorescence correlation spectroscopy (FCS).[3] **P1** was distributed as a single molecule in water (1×10^{-7} mol/L) as shown by FCS measurement. The UV/vis absorption and emission spectrum of **P1** were measured in water (Figure 1B). The emission maximum was recorded at 611 nm (Figure 1C), which is generally considered advantageous for biological imaging experiments.[4] Using Cresyl Violet in methanol ($\Phi_f = 0.54$) as a reference chromophore,[5] a fluorescence quantum yield (Φ_f) of 0.11 was obtained for **P1** in water. To test the photostability of this macromolecule, the aqueous solution of **P1** (1×10^{-6} mol/L, volume = 1 cm^2 × 3 cm) was irradiated under UV light (40 W, 350 nm) for 3 days (20 cm distance between solution and UV lamp) or exposed to natural light for 2 weeks. The intensity of the UV/vis absorption and the fluorescence of **P1** in water remained almost unchanged.

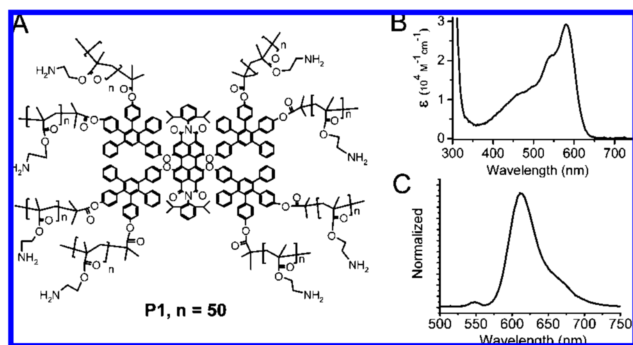

Figure 1. Structures of **P1** (A), absorption (B), and normalized emission spectrum (C) of **P1** in water (1×10^{-6}M).

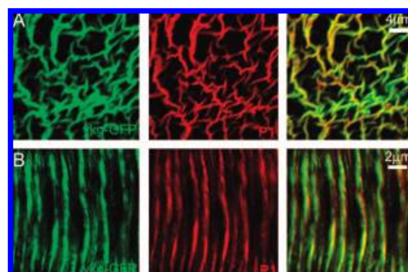

Figure 2. Confocal images of ECM staining with **P1** staining (red, central column) of ECM in fixed wing epithelium (A) and in living tracheal epithelium (B) of vkg-GFP transgenic larvae. Anti-GFP staining (A) and GFP fluorescence (B) are in green (left column). **P1** staining was for 30 min in A and 45 min in B. Merged channels are shown in the right column.

Drosophila is a popular model system in animal research. The main constituents of the ECM are well conserved in evolution. It has been shown in numerous studies that cell-biological findings in *Drosophila* can be extrapolated to mammals. To determine the binding property of **P1** on animal tissues, the histologically well-characterized *Drosophila* wing imaginal disk was dissected and fixed for double staining with **P1** and antibody. The ECM can be labeled using anti-GFP antibody against ECM-localized GFP fusion proteins, such as Vkg-GFP in transgenic larvae.[6] In high resolution confocal microscopic sections through the ECM plane, **P1** staining nearly fully overlapped with Vkg-GFP revealing the ECM micro-network structure with the same specificity as vkg-GFP (Figure 2A).

P1 was also tested on living tissue without prior fixation. Because the Vkg-GFP autofluorescence was weak in the wing imaginal disk, the more strongly fluorescent *Drosophila* larval tracheal tissue was selected for in vivo staining. **P1** efficiently stained the tracheal ECM when the tissue was cultured for 45 min in Schneider's insect medium containing 0.01% **P1** (Figure 2B and S-Figure 5).

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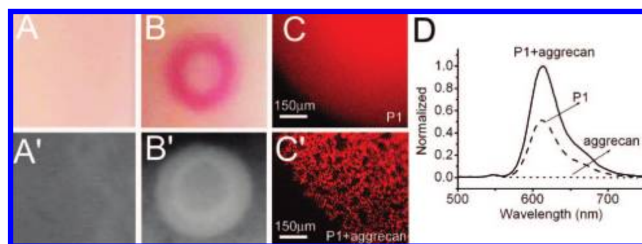

Figure 3. Characterization of the **P1**/aggrecan interaction. (A and B) Dot blotting assay for **P1**/protein binding. **P1** does not accumulate on the dot of collagen (A, under visible light; A′, under UV light), but accumulates on the dot of aggrecan (B, under visible light; B′, under UV light). (C) Vapor diffusion assay for the **P1**/aggrecan interaction. Fluorescent images of **P1** alone (C) and **P1**/aggrecan complex (C′) in buffer solution. (D) Spectral analysis of the **P1**/aggrecan interaction.

Compared to antibody staining procedures, which may require several days, the staining with **P1** was much faster. These results demonstrate the specific staining of the ECM by **P1** and indicate that there might be a direct binding between negatively charged ECM components and the positively charged **P1**.

In order to test the above assumption and to investigate the binding characteristics of **P1** and some main ECM components, several techniques were applied such as dot blotting, multiple substrate array (MSA), vapor diffusion assay, and spectral analysis. Two characteristic ECM proteins, neutral bovine collagen and acidic aggrecan, were tested first. Aggrecan is a major structural proteoglycan of cartilage ECM with a 210−250 kDa core protein to which 100−150 chondroitin sulfate and keratan sulfate chains are attached (S-Figure 4C).[7] Tissue collagen occurs as big fibers composed of trimeric largely uncharged α-chains, which are stabilized by cystine bonds. The primary sequence of the collagen α-chains consists of a repeating tripeptide made up of glycine, proline, and hydroxyproline such that collagen has little net charge.[8] In the dot blotting assay performed on positively charged Nylon membranes, negatively charged aggrecan but not neutral collagen led to **P1** accumulation on the protein dot, which could be visualized under both visible and UV light (Figure 3A and B). Moreover, other negatively charged binding partners in ECM were also identified by MSA (S-Figure 6 and Supporting Information text).

In a vapor diffusion assay, **P1** and aggrecan were mixed into a buffer where they formed complexes, in contrast to the evenly distributed **P1** alone (Figure 3C and C′). A direct interaction between **P1** and aggrecan was also revealed by spectral analysis. Compared to the isolated compounds (Figure 3D), the mixture showed a slight bathochromic shift of 3 nm of the emission maximum and a significantly increased emission intensity (Φ_f = 0.22). All these assays showed a strong interaction between **P1** and the main negatively charged ECM components, supporting the concept of a direct binding between negatively charged ECM components and the positively charged **P1** macromolecule bearing multiple amines.

We here showed that a positively charged fluorescent core−shell macromolecule bearing multiple primary amine groups successfully visualized the microstructure of ECM in both fixed and living preparations by strongly binding to its negatively charged constituents. The dye significantly increased in emission intensity after binding to ECM components and was compatible with antibody staining, making it useful for multiple channel fluorescence imaging. Due to its water solubility and optical properties as well as biological specificity, this novel fluorescent dye provides an attractive tool for specifically labeling the ECM in life science research.

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Supporting Information Available: Complete ref 6, experimental procedures, characterization of the macromolecule **P1**, supplementary figures and text. This material is available free of charge via the Internet at http://pubs.acs.org.

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