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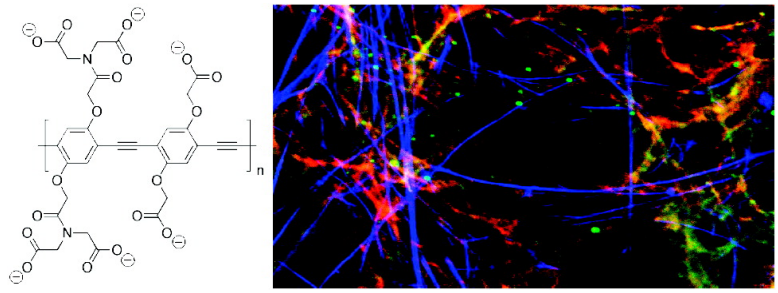
J. Am. Chem. Soc., **2008**, 130 (25), 7851-7853 • DOI: 10.1021/ja8007402 • Publication Date (Web): 29 May 2008



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Published on Web 05/29/2008

Molecular Recognition Based on Low-Affinity Polyvalent Interactions: Selective Binding of a Carboxylated Polymer to Fibronectin Fibrils of Live Fibroblast Cells

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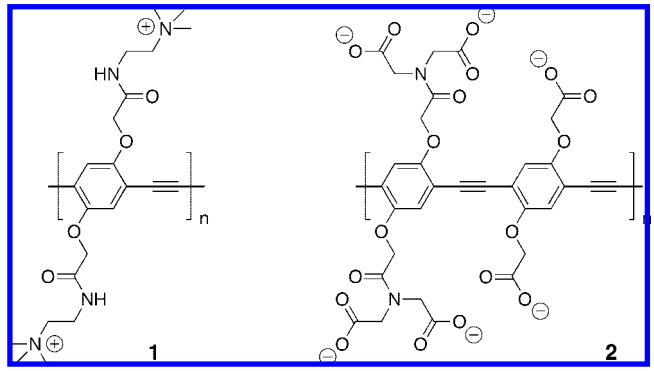
Polyvalent interactions are prevalent throughout nature and play a critical role for maintaining many biological functions including cell−cell recognition, cell adhesion, cell proliferation, signal transduction, and gene regulation.[1] Synthetic ligands containing multivalent recognition elements may selectively bind to cell-surface receptors and other components of the extracellular matrix and thus harbor the potential to act as selective inhibitors or effectors of these processes.[2] Compared to their monovalent counterparts, the selectivity and affinity of polyvalent ligands are often superior,[3] therefore offering promising opportunities for the development of new target-specific drugs. For example, multivalent ligands have been developed to inhibit binding of pathogens to host cells,[4] to selectively target tumor cells,[5] or to stimulate immune responses.[6] Polyvalent interactions have been also exploited for designing sensitive analytical reagents. In particular, multifunctionalized synthetic polymers have demonstrated great versatility for the detection of a wide range of analytes, including DNA, metal ions, nitric oxide, lectins, proteins, and bacteria.[7]

The majority of polyvalent synthetic ligands are composed of recognition elements that display already significant selectivity toward the target site as single isolated moieties. The goal of this study was to explore whether a ligand offering multiple *nonspecific* interactions might lead to selective recognition of components in the extracellular matrix (ECM) of live cells. To assemble the individual binding elements in an organized fashion, we utilized a linear, conjugated polymer (CP) as a scaffold. CPs such as poly(*p*-phenyleneethynylene)s (PPEs), polyfluorenes, or poly(*p*-phenylenevinylene)s are intrinsically fluorescent[8,9] and can be readily visualized by means of fluorescence microscopy. Furthermore, the addition of polar groups render CPs water-soluble without compromising their fluorescence properties. For this study, we utilized poly(*p*-phenyleneethynylene) as the backbone, which was functionalized either with positively charged tetraalkylammonium groups (PPE **1**)[7j] or with negatively charged carboxylates (PPE **2**)[7j,9] as nonspecific low-affinity binding elements.

As evident from Figure 1, the two polymers behaved distinctly different when added to live NIH 3T3 fibroblast cells in growth medium (DMEM) at 37 °C for 4 h. While the cationic PPE **1** yielded a punctate staining pattern reminiscent of endocytic vesicles, PPE **2** carrying a net negative charge showed a characteristic filamentous extracellular staining pattern.

The endocytosis of polycationic molecules, in particular, derivatives of the cell penetrating peptides HIV-Tat or polyarginine, is well-documented.[10] Depending on the cargo, different uptake mechanisms might be involved; however, internalization of the cationic molecules is most likely initiated through interaction with negatively charged proteoglycans located within the extracellular matrix. Interestingly, uptake of PPE **1** was only partially complete within 30 min and required at least 4 h incubation time for full



internalization (Supporting Information), suggesting a mechanism that is different from receptor-mediated endocytosis. Prolonged incubation for 24 h did not lead to additional changes (Figure 1, right). Furthermore, the punctate staining pattern of **1** did not colocalize with the subcellular distribution of common endocytic markers such as the transferrin receptor, mannose-6-phosphate receptor (recycling and late endosomes), or lamp1 and lysotracker red (for lysosomes and acidic compartments, see Supporting Information). In contrast, extracellular staining with PPE **2** occurred within minutes, while prolonged incubation over a period of 24 h led to almost complete internalization with a punctate staining pattern similar to that of PPE **1** (Figure 1, right).

To elucidate the nature of the interaction partner of the anionic PPE **2**, we performed a series of histochemical and immunofluo-

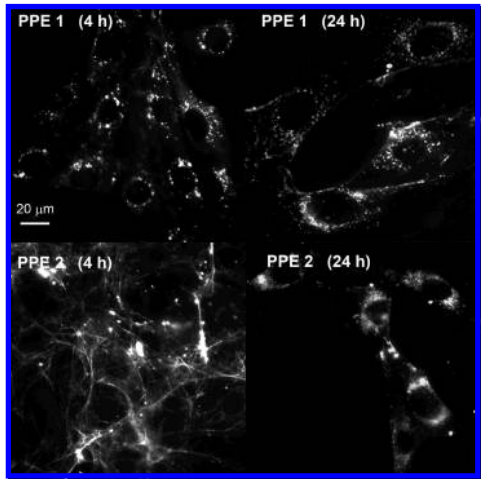


Figure 1. Time dependence of the interaction of PPEs with live mouse fibroblast cells (NIH 3T3). Fluorescence micrographs upon incubation with PPE **1** (top row) or PPE **2** (bottom row) at 37 °C for 4 and 24 h (25 μM polymer in DMEM).

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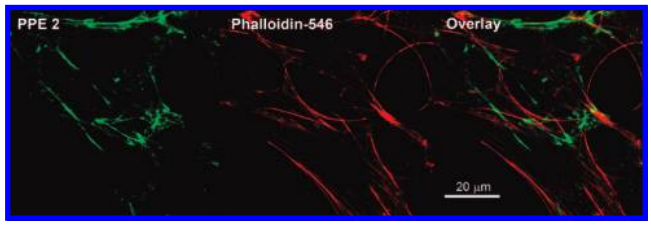

Figure 2. Immunofluorescence colocalization of **2** with phalloidin-546, a histochemical reagent for F-Actin. From left to right: Fluorescence micrograph of NIH 3T3 cells stained with PPE **2** (green), phalloidin-546 (red), and false color overlay of **2** and phalloidin-546.

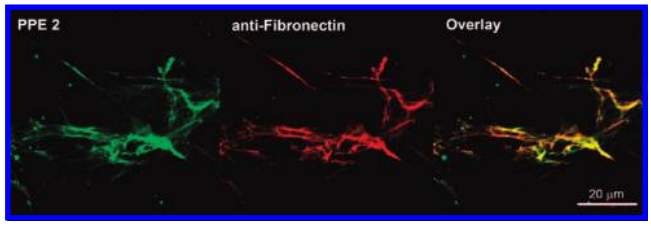

Figure 3. Immunofluorescence colocalization of **2** with an antibody specific for fibronectin. From left to right: Fluorescence micrograph of NIH 3T3 cells stained with PPE **2** (green), anti-fibronectin (red), and false color overlay (areas of colocalization are revealed in orange/yellow).

rescence studies. In a first experiment, we co-incubated live 3T3 cells with **2** and phalloidin-546, a specific histochemical reagent for visualizing the distribution of filamentous actin (F-Actin). As shown in Figure 2, only few areas of overlap were observed; however, a closer inspection of the dual fluorescence micrograph showed that the PPE staining pattern appeared to some degree aligned with F-Actin.

We thus hypothesized that **2** might bind to fibronectin, an extracellular matrix protein, which is known to interact with actin filaments at specific locations within the ECM.[15] Immunofluorescence staining using a commercially available antibody against fibronectin revealed an almost perfect colocalization with PPE **2**, as demonstrated by the yellow areas in the false color confocal micrograph (Figure 3). For additional comparison, a triple fluorescence micrograph showing the subcellular localization of F-Actin, fibronectin, and PPE **2** is provided in the Supporting Information.

While the immunofluorescence experiments demonstrate microscopic colocalization of PPE **2** with anti-fibronectin, the spatial resolution is insufficient to demonstrate binding on a molecular level. It is conceivable that the polymer might associate only indirectly with the fibrils through another protein. To directly probe the interaction of PPE **2** with fibronectin, we performed an in vitro binding assay. Fibronectin was adsorbed at different densities on the glass surface of a 96-well plate, then exposed to increasing concentrations of PPE **2**, and upon equilibration for 1 h washed to remove unbound polymer. The degree of complex formation was then directly assessed with a microplate reader on basis of the fluorescence intensity of fibronectin-bound polymer. As illustrated with Figure 4, the fluorescence increased not only with increasing polymer concentration but also as a function of the fibronectin surface density. In absence of fibronectin, PPE **2** showed only little adsorption on the glass surface (control). To test for nonselective binding to a protein other than fibronectin, we adsorbed bovine serum albumin (BSA) on the glass surface. Titration with increasing concentrations of PPE **2** showed no interactions beyond the level of the control in absence of a protein (see Supporting Information).

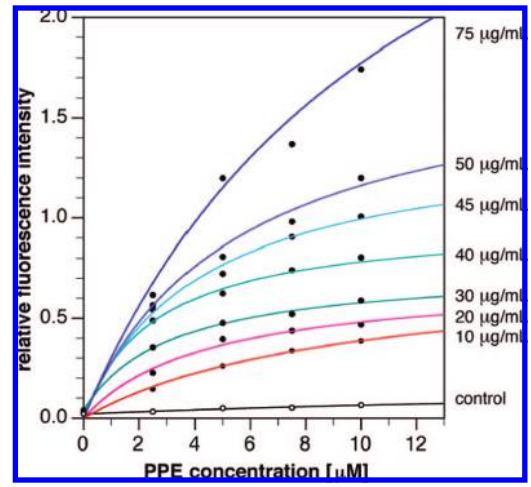

Figure 4. Binding assay of PPE **2** with surface-adsorbed fibronectin. The glass surface of a 96-well plate was incubated with different concentrations of fibronectin as indicated on the right side of the graph. Changes in fluorescence intensities (λ_{ex} = 410 nm, λ_{em} = 460 nm) with varying concentrations of **2** (based on monomer M_w) were measured with a plate reader, and the resulting binding isotherms were fitted with eq 1). Control: fluorescence change in the absence of fibronectin.

Table 1. Dissociation Constants for the Interaction of PPE **2** with Fibronectin Adsorbed to a Glass Surface[a]

fibronectin[b] (μg/mL)	K_D[c] (μM)	SD[d] (μM)	F_{max}
10	7.4	2.1	66 (±12)
20	4.5	0.3	67 (±10)
30	2.3	0.2	68 (±1)
40	2.6	0.1	98 (±1)
45	4.2	0.3	141 (±4)
50	5.4	0.1	179 (±2)
75	11.8	0.6	387 (±13)
100	11.5	0.5	389 (±12)

[a] At pH 7.2, 10 mM PIPES buffer, 25 °C. [b] Concentration of surface coating solution. [c] Dissociation constant based on monomer M_w. [d] Standard deviation of K_D obtained from nonlinear least-squares fit with eq 1.

Assuming full equilibration and a slow dissociation rate of polyvalently bound polymer,[11] the changes in fluorescence intensity F can be interpreted as Langmuir isotherms according to eq 1[12]

$$F = \frac{F_{max}[P]_0}{K_D + [P]_0} \quad (1)$$

where F_{max} is the maximum fluorescence intensity (a measure of the binding capacity), $[P]_0$ is the total polymer concentration, and K_D is the equilibrium dissociation constant of the polymer−fibronectin complex adsorbed on the glass surface.

A comparison of the dissociation constants K_D obtained from analysis of each binding isotherm revealed a rather narrow distribution (Table 1). At a fibronectin coating concentration of 100 μg/mL, the surface density reached saturation as reflected by the converging F_{max} values. Averaging over the intermediate fibronectin coating concentration range of 20−50 μg/mL, an apparent dissociation constant of 3.8 ± 1.3 μM was calculated (based on monomer molecular weight). Considering the average molecular weight M_n = 36 kDa of polymer **2**,[9] the apparent dissociation constant of the fibronectin−polymer complex is approximately 100 nM, a value that is in agreement with the polyvalent nature of the interaction. It is noteworthy that the avidity of the polymer−fibronectin complex lies in a similar range compared to the multivalent carbohydrate−lectin interactions.[13]

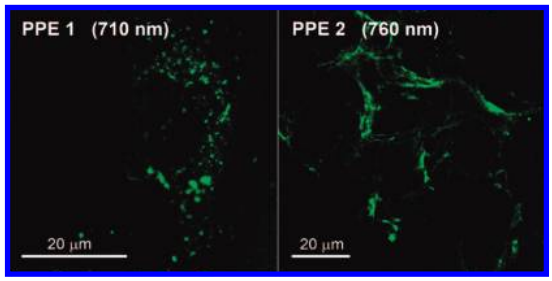

Figure 5. Two-photon excitation microscopy of PPE derivatives interacting with live mouse fibroblast cells. Cells were incubated with PPE **1** (left, λ_{ex} = 710 nm) or PPE **2** (right, λ_{ex} = 760 nm) in DMEM at 37 °C for 4 h.

While we can only speculate about the nature of the fibronectin binding sites that interact with PPE **2**, recent structural data of several fibronectin domains revealed an extended area of positive charge within FN repeats 12−14, a location that is involved in binding of polyanionic heparin.[14] Repeating the above in vitro assay in the presence of 100 μg/mL heparin sulfate yielded indeed a marked reduction of PPE **2** binding to fibronectin (Supporting Information), thus supporting this hypothesis.

Encouraged by these results, we tested next the performance of the PPEs as fluorescent dyes for two-photon excitation microscopy (TPEM). Improved depth penetration in tissue samples, significantly reduced background excitation of endogenous molecules, as well as reduced phototoxicity combined with a small excitation volume suitable for 3D imaging have rendered TPEM the preferred imaging modality over conventional confocal laser scanning microscopy. The brightness of each PPE was evaluated over a range of wavelengths in chemically fixed cells (data not shown), revealing the optimum excitation wavelength of 710 nm for PPE **1** and a slightly longer wavelength of 760 nm for the negatively charged PPE **2**. Both polymers showed bright fluorescence emission combined with good photostability that renders them well-suited for TPEM (Figure 5).

To further evaluate the applicability of PPE **1** and **2** in live cell studies, we assessed their acute toxicity as a function of PPE concentration and incubation time using the trypan blue exclusion assay. According to these studies, the cell viability remained unchanged within 6−8% of untreated cells up to a concentration of 50 μM and for as long as 24 h. Only when incubated at a concentration of 100 μM PPE over 24 h, the viability decreased by approximately 10% compared to untreated cells (see Supporting Information).[5b]

In conclusion, the polyvalent nature of carboxylated PPE **2** led to selective recognition of the extracellular matrix protein fibronectin. This observation is particularly intriguing considering the intrinsically low affinity of a single carboxylic acid moiety toward a potential target site and the simple topology of the nonchiral, linear polymer backbone. Furthermore, the internalization of the anionic polymer **2** upon prolonged incubation is noteworthy since negatively charged molecules are typically not readily transported into live cells.

Fibronectin participates in an array of essential biological processes and is also vital for the progression of numerous diseases, including cancer cell survival as well as bacterial or viral infections. This relevance has particularly sparked interest for elucidating the nature and role of its many binding partners and the mechanism of fibronectin fibril assembly.[15] Given the biological importance of fibronectin, its selective recognition by a structurally uniform polymer underscores the utility of weak nonspecific polyvalent interactions for the design of new synthetic ligands.

Acknowledgment. Financial support from the National Institutes for Health (GM072021, R.L.M., C.J.F.) and the Department of Energy (DOE-DE-FG02-04ER46141, U.H.F.B., R.L.P., I.-B.K.) is gratefully acknowledged. We also thank Prof. Harold P. Erickson for helpful advice.

Supporting Information Available: Detailed experimental procedures, immunofluorescence and histochemical micrographs, and cell viability data. This material is available free of charge via the Internet at http://pubs.acs.org.

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JA8007402