

Conjugated Polyelectrolyte-Antibody Hybrid Materials for Highly Fluorescent Live Cell-Imaging

Kangwon Lee, Jiseok Lee, Eun Jeong Jeong, Adam Kronk, Kojo S. J. Elenitoba-Johnson, Megan S. Lim, and Jinsang Kim*

Convenient and cost-effective methods for bioimaging in real time particularly with high sensitivity are highly desired in medical diagnosis, identification of cancer cells, immunofluorescent techniques, catalytic pathway monitoring, drug delivery monitoring through membrane or cytoplasm, and identification of genetic mutations in cells.^[1] Conventional cell staining techniques for immunofluorescence microscopy require time and cost consuming multiple steps in sample preparation such as, fixation of cells, blockings, and primary and secondary antibodies treatments. By contrary, direct labeling not only greatly reduces required preparation steps but also, and more importantly, can avoid the common problems of cross-reactivity and high-level background. Small fluorescent molecules and inorganic quantum dots have been extensively studied in labeling biological entities such as bacteria, viruses, cells, and tissues.^[2] However, small organic fluorescent molecules often suffer from photo-bleaching and the much more stable inorganic quantum dots such as CdSe and CdTe are not free from potential cytotoxicity due to possible heavy metals leaching from the nanoparticles. Another potentially critical issue in the application of inorganic quantum dots to *in vivo* bioimaging is their aggregation resulting from the disruption of the passivation layer of the quantum dots induced by environmental change.^[2b,2i,2o,3] Therefore, there is a great need to devise a bioimaging method that is simple, nontoxic, and can provide high sensitivity.

Conjugated polyelectrolytes (CPEs) are conjugated polymers having ionic or non-ionic water soluble side chains for the solubility of the polymers in water. The molecular design of signal amplifying conjugated polymer-based biosensor and highly fluorescent and water-soluble CPEs for novel biosensors are a

topic of much scientific interest.^[4] The large molecular weight and a high extinction coefficient of CPEs provide a unique energy harvesting property to the CPEs.^[5] Therefore, if the side chain of the CPEs is rationally designed to efficiently prevent aggregation of the hydrophobic backbone of CPEs in water, CPEs become a bright emitter in aqueous environment. Also, a hydrophilic ethylene oxide chain was recently reported to prevent non-specific binding to cells or tissue and a good candidate to sheathe hydrophobic dyes or nanoparticles. Abundant side chains and the two chain ends of CPEs are available to introduce a reactive functional group for additional modifications and bioconjugation.^[4a] Recently, interesting reports showing cell staining using CPEs containing amine groups have been published.^[6] However, there hasn't been systematic investigation about using CPE-antibody conjugates for cell staining even though more sensitive immunofluorescence staining could be achieved.

Here, we report novel CPE-antibody conjugates for fast, convenient, and highly sensitive live cell imaging. It is advantageous to use primary antibodies directly labeled with a fluorophore without secondary antibody. Since the secondary antibody step is omitted, the direct imaging is relatively quick, and avoids potential problems of cross-reactivity of the secondary antibody with components in the antigen sample. Therefore, the hybrid CPE-antibody conjugates were prepared by means of direct bioconjugation between an antibody (CD3 or CD20) and a conjugated poly(*p*-phenyleneethynylene) (PPE) derivative (PPE1 or PPE2) having blue or red fluorescent emission (**Figure 1**). CD20 is an antibody which selectively recognizes B-cells only while CD3 will bind to T-cells specifically. CD3 is expressed by thymocytes in a developmentally regulated manner and by all mature T cells. CD20, also known as membrane-spanning 4-domain, group A, member 1[MS4A1], encodes a B-lymphocyte membrane molecule which plays an important role in the development and differentiation of B-cells into plasmocytes.^[7] The CD20 molecule is clinically significant as target of monoclonal antibodies, which are all active agents in the treatment of B cell lymphomas and leukemias.^[8] The two CPEs, PPE1 and PPE2, were prepared by Pd-catalyzed polymerization and have carboxylic acids on the side chains or at the two chain ends of the CPE. The CPE was covalently linked to the antibody as a macromolecular fluorescent reporter through the carbodiimide chemistry between the carboxylic acid of the CPE and amine groups of the antibody. By conducting systematic sensitivity, cross-selectivity, cell viability, and dilution tests, we demonstrate selective and specific staining of both peripheral blood and cell line-derived B and T lymphocytes using CPE-conjugated antibodies to CD20 and CD3. The developed CPEs and the method

Dr. K. Lee,^[†] Dr. E. J. Jeong, Prof. J. Kim
Department of Materials Science and Engineering
University of Michigan
2300 Hayward St. Ann Arbor, MI 48109, USA
E-mail: jinsang@umich.edu

Dr. J. Lee
Macromolecular Science and Engineering
University of Michigan
2300 Hayward St, Ann Arbor, MI 48109, USA

A. Kronk, Prof. K. S. J. Elenitoba-Johnson, Prof. M. S. Lim
Department of Pathology
University of Michigan
1301 Catherine St. Ann Arbor MI 48109, USA

[†] Present address: Samsung Information Systems America, Inc.,
1 Cambridge Center #702, Cambridge, MA 02142, USA



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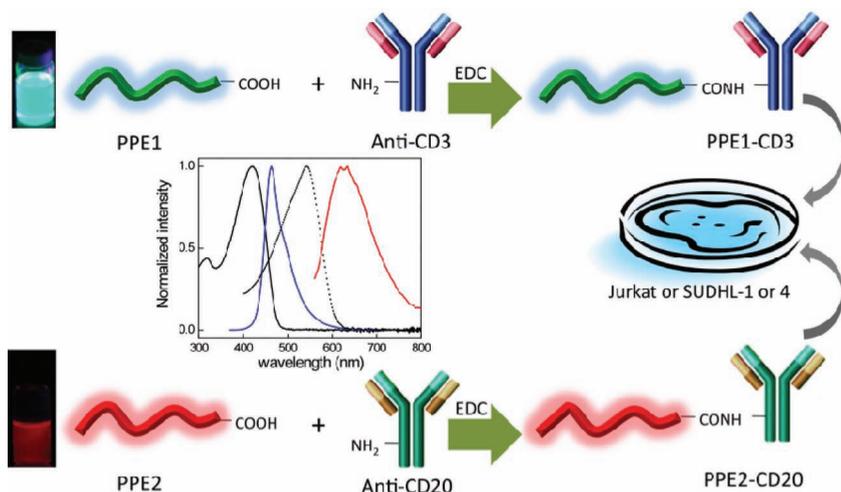


Figure 1. Overall strategy of cell imaging with water-soluble fluorescent polymer-antibody conjugates. (Inset) The absorption and photoluminescence spectra of PPE1 (UV:black/PL:blue) and PPE2 (UV:green/PL:red). PPE1 (100 nM) and PPE2 (150 nM) in deionized water was excited at 365 nm and 540 nm, respectively.

for bioconjugation of the CPEs with antibodies virtually can be applied for the direct labeling of any biological molecules.

Monomer and polymer synthesis for PPE1 and PPE2 are described in supporting information. Figure 1 (inset) shows the absorption and emission spectra of PPE1 and PPE2 in deionized water. PPE1 and PPE2 showed blue-green emission at 460 nm and red emission at 630 nm, respectively. The absolute quantum yield of PPE1 in water measured in an integrating sphere was in the range of 38–57% depending on the concentration (10^{-4} – 10^{-7} M). On the other hand, the quantum yield of PPE2 (0.15 μ M) was $1.1 \pm 0.6\%$ in water.

Labeling of proteins with a chromophore/fluorophore is a universal method in colorimetric assays and immunofluorescence. However, if many chromophores/fluorophores are attached to an antibody or enzyme for a stronger signal such labeling can negatively affect the function of the antibody and the enzyme. The molar mass of CPE is much larger than that of small chromophores and fluorophores. Therefore, by labeling an antibody with CPE instead of small molecular chromophores and fluorophores a much brighter CPE-antibody can be prepared and can achieve much more sensitive assays without giving any negative effects on the function of the antibody. With this in mind, we investigated a covalent conjugation strategy in which carboxylic acid side chains of the CPE and lysine amines of the antibody are reacted to form an amide linkage. Using commercially available reagents (EDC and sulfo-NHS), a succinimide functionality was introduced to the carboxylated CPE via carbodiimide chemistry to facilitate the amide bond formation with an amine group of the antibody. We prepared CPE-antibody conjugates having

different numbers of CPE per antibody by controlling the stoichiometric amount of CPE per antibody during the bioconjugation reaction. The resulting CPE-antibody conjugates were characterized by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The SDS-PAGE result of PPE1-CD3 conjugates and PPE2-CD20 conjugates are shown in Figure 2. We used the heat-induced denaturation preceding gel electrophoresis (100 °C) to consider only covalently bound CPE-antibody by minimizing the possible non-specific binding of CPE to the antibody. On the lane A of Figure 2a, only pure CD3 was run as a reference. Two bands are shown in the lane A. One is corresponding to the heavy chain (60 kDa) of CD3 and the other is the F_{ab} light chain (23 kDa) of CD3, an IgG2-type antibody. On the lanes B through E we ran the resulting PPE1-CD3 conjugates prepared with different amount of PPE1 per CD3 as indicated in the figure caption.

After the conjugation with 5 times excess PPE1 (lane B), the heavy chain band at 60 kDa disappeared and instead a new band over 180 kDa appeared. Considering that the number average molecular weight of PPE1 is 73 kDa the location of the PPE1-CD3 conjugates in the gel is reasonable. The broad feature and the smearing of the PPE1-CD3 bands are likely due to the combination of the polydispersity of PPE1 and the distribution of the number of the bound PPE1 per CD3. As the amount of PPE1 added to the bioconjugation increased from the lane C to E, the band of the PPE1-CD3 conjugate gradually moved to a higher molecular region. This result indicates that PPE1 is predominantly conjugated to the heavy chain. SDS-PAGE result for

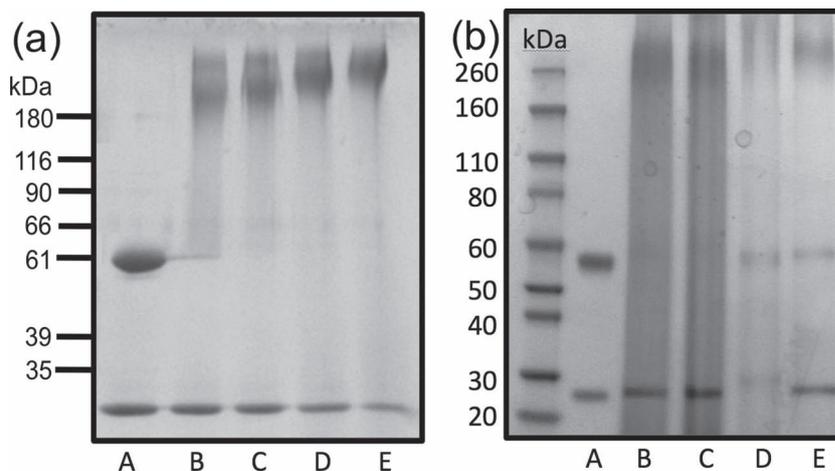


Figure 2. SDS-PAGE analysis with 10% Tris-Glycine Gel (PPE1) and 4-12% Bis-Tris Gel (PPE2); (a) CD3 and PPE1-CD3 conjugates having varying stoichiometric amount of PPE1 per antibody (Lane A: only CD3 and lane B-D correspond to 5:1, 10:1, 50:1, 100:1 (PPE1:CD3 in mole), respectively. Coomassie Blue staining used. Bands located at the bottom of the gel are corresponding to the F_{ab} light chain of 23 kDa), (b) CD20 and PPE2-CD20 conjugates (Lane A: only CD20, lane B-E correspond to 100:1, 10:1, 1:10, 1:100 (PPE2:CD20 in mole), Coomassie Blue staining used. Bands located at the bottom of the gel are corresponding to the F_{ab} light chain of 25 kDa).

the PPE2-CD20 conjugates also shows that a new band at each lane appeared at the region corresponding to 260 kDa (Figure 2b). When excess amount of PPE2 was added in conjugation step (lane B and C), a long tailing band was observed through lane B and C coming from unbound PPE2 stained in the gel. Unlike PPE1-CD3, PPE2-CD20 looks to have the same molecular weight regardless of the amount of PPE2 added. This is because that PPE2 has only two carboxylic acids at the ends of the polymer chains for conjugation with CD3. When the amount of CD20 increased excessively (lane D and E), we observed CD20 unbound, which is expected to happen during conjugation. Therefore, PPE1 and PPE2 are covalently bound to CD3 and CD20, respectively. For the cell staining study presented in this contribution we used the bioconjugation product from 10:1 (PPE:antibody) ratio.

We studied the selectivity of the CPE-antibody conjugates to see whether the CPE tethering to the antibodies affects the specificity of the antibodies. Comparison between the DIC images and the fluorescence images in the Figure 3a and Figure 3b clearly show that the Jurkat cells were selectively stained with PPE1-CD3 as expected while the B cells (SUDHL-4) were not stained by the conjugates, confirming the maintained specificity. Fluorescence images through confocal laser scanning microscope showed that cell membranes were selectively stained by polymer-antibody conjugates, verifying that epitope recognition of an antibody on plasma membrane was not hindered by a polymer chain (Figure S3 in the supporting information). The few blue emissive dots in the fluorescence image of Figure 3b are likely due to non-specifically bound PPE1 aggregates on the substrate that were not removed somehow through the purification step after the bioconjugation. The basis of this postulation is the fact that in the DIC image there is no cell at the locations where the blue dots are observed in the fluorescence image. Similarly, SUDHL-4 was selectively stained by PPE2-CD20 but SUDHL-1 (T-cell) was not stained by the conjugate (Figure 3c and d). These results imply that the CPE tethering to the antibodies does not affect the specificity of the antibodies. The primary purpose of CPEs in the development of CPE-antibody conjugates is to achieve improved sensitivity in live cell imaging. In Figure 4, we examined the sensitivity of our CPE-antibody conjugates by comparing the fluorescence intensity between Jurkat cells stained with PPE1-CD3 and FITC-labeled CD3, respectively. A larger size and a high extinction coefficient of PPE1 produced much stronger fluorescence emission from PPE1-CD3 than that of

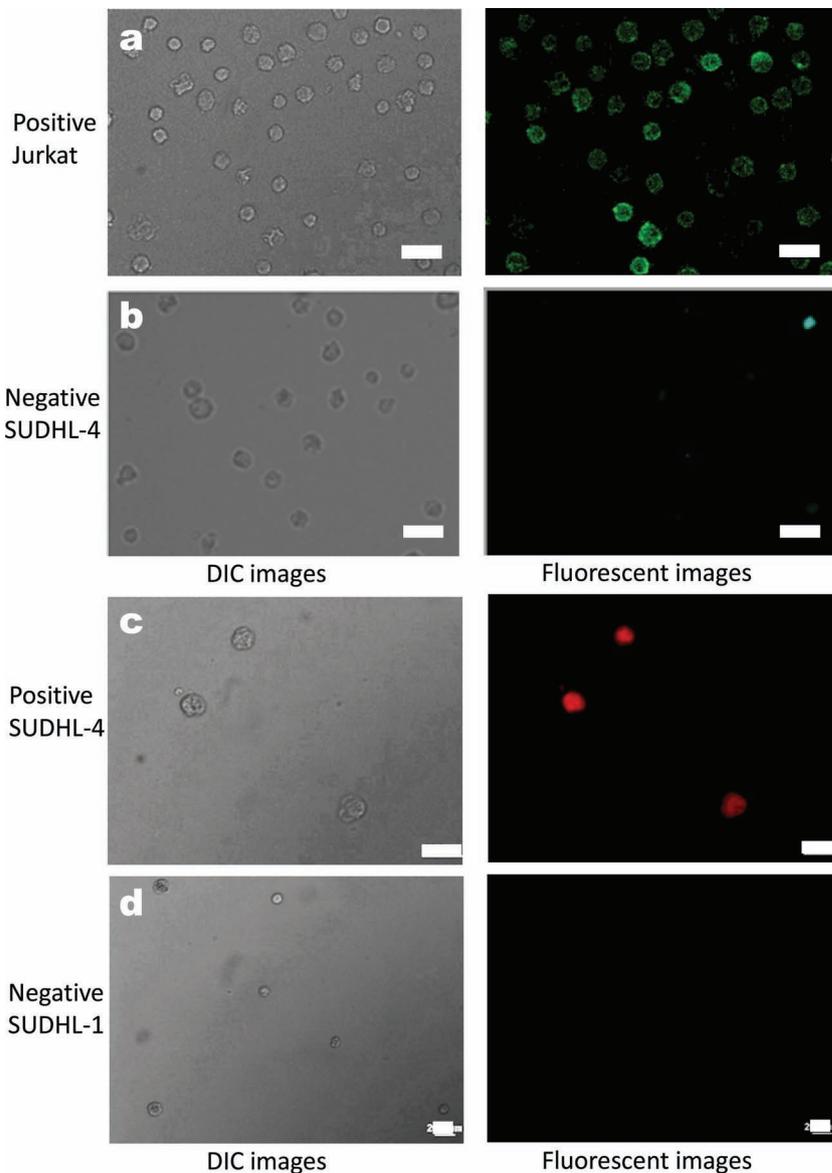


Figure 3. Differential interference contrast (DIC) images and fluorescence microscope images of live cells after the incubation with PPE1-CD3 (a: Jurkat and b: SUDHL-4) and PPE2-CD20 (c: SUDHL-4 and d: SUDHL-1) for 30 min. DIC images are shown in the left column and fluorescence images are in the right column. CD3 is specifically expressed on T-cells (Jurkat and SUDHL-1) while CD20 is specifically expressed on B-cells (SUDHL-4). The fluorescence images for a and b were obtained at the excitation wavelength of 470 nm and the emission cut-off of 500 nm and the images for c and d were from 560 nm excitation and 620 nm for emission. Scale bar: 20 μm .

FITC-CD3 and visualized Jurkat much more clearly. The fluorescence intensity of PPE1-CD3 was calculated to be 6.4 times larger than that of FITC-labeled CD3. To compare the fluorescence intensity, the emission intensity of a given area of the images was measured and divided by the number of cells in the area. Therefore, the CPE-antibody conjugates can provide more sensitive live-cell imaging.

Having established that PPE1-CD3 and PPE2-CD20 can effectively target and stain the membranes of Jurkat T-cells and B-cells, respectively, we then investigated the cross-selectivity

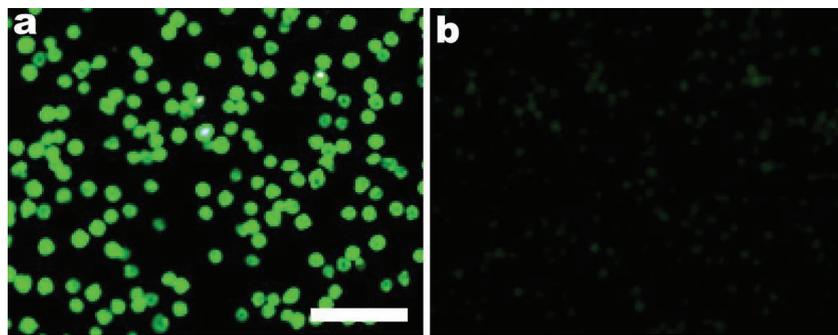


Figure 4. Fluorescence microscope images of live Jurkat cells stained with (a) PPE1-CD3 and (b) FITC-labeled CD3. All fluorescence images were obtained in the same microscope conditions with the same exposure time for direct sensitivity comparison. A GFP long pass emission filter (>500 nm) and an excitation filter (450–490 nm) were used. Scale bar: 50 μm .

of the CPE-antibody conjugates in the presence of mixed cells. Equal amount of Jurkat and SUDHL-4 were mixed together as suspension in a buffer and PPE1-CD3 and PPE2-CD20 conjugates were then added to the cell suspension. Jurkats were selectively stained with PPE1-CD3 conjugates whereas B cells were stained with exclusively PPE2-CD20 (Figures S5(a) and S5(b) in supporting information). The fluorescence image in Figure S5(c) clearly demonstrated that our CPE-antibody conjugates have excellent cross-selectivity and are suitable for immunofluorescence techniques. We also did the dilution tests to find if our CPE-antibody conjugates can be applicable to quantitative cell counting and cell sorting. Mixed cells of SUDHL-4 and Jurkat having different mixing ratios were stained with the CPE-antibody conjugates. The ratio (the number of stained SUDHL-4/total number of stained cells) is plotted against the cell mixing ratio in Figure 5. The number of the stained cells was counted from fluorescence microscope images of each sample by using a hemacytometer. The linear correlation curve implies that CPE-antibody conjugates are suitable for quantitative cell counting and cell sorting.

The cytotoxicity of the CPEs to SUDHL-4 and Jurkat was investigated with various concentrations of PPE1 and PPE2

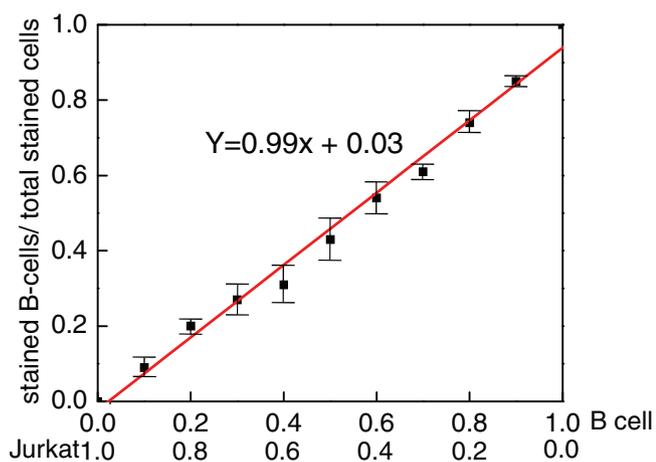


Figure 5. The correlation between the ratio (the number of stained SUDHL-4/total number of stained cells) and the cell mixing ratio.

(nM– μM). We incubated living cells, Jurkat and SUDHL-4, in RPMI1640 cell media together with PPE1 for 72 h and analyzed the viability and proliferation of the cells. As a control, the same cells were also incubated in the same conditions without adding PPE1. Surprisingly, we hardly observe any dead cell even from the batches incubated with 1×10^{-6} M PPE1, the highest concentration. This means that the cells have over 99.9% viability even in micromolar concentration regime. At a concentration of 1×10^{-6} M of PPE1, the proliferation of SUDHL-4 and Jurkat slightly decreased to 86% and 80% compared with the control cells after 72 h of incubation (Figure 6a and 6b). Total number of SUDHL-4 and Jurkat

cells after incubation for 72 h increased by 10 and 15 times of the number of the initial cells, respectively (Figure 6c). The cell doubling time of SUDHL-4 and Jurkat was 21 and 18 h, respectively.^[9] The same test was conducted with PPE2 and no death cell was observed in the presence of PPE2 (Figure S6 in supporting information). Fluorescence microscopy was also applied after incubating SUDHL-4 with PPE1 to examine the interaction between PPE1 and the cell surface. Even though a negatively charged CPE showed staining of fibroblast cells in a recent report,^[6a] we did not observe any PPE1 emission from SUDHL-4 after incubation with PPE1 and subsequent washing, suggesting that there is a negligible non-specific binding between PPE1 and the cells (Figure 6d). There is a green fluorescent spot in Figure 6d but by comparing the image and the inset we can see that where the fluorescent spot was observed there was no cell.

We have demonstrated that rationally designed conjugated polyelectrolytes can be covalently attached directly to an antibody as a fluorescent reporter molecule without affecting the recognition specificity of the antibody. Two fluorescent and water-soluble CPEs having blue (PPE1) and red (PPE2) emission, respectively, were synthesized and bioconjugated with CD3 and CD20, respectively, to form CPE-antibody conjugates. PPE1-CD3 showed excellent specificity toward T-cells (Jurkat) and PPE2-CD20 selectively bound to B-cells (SUDHL-4). Due to the high extinction coefficient of CPEs, the developed CPE-antibody conjugates showed much higher sensitivity in the live cell imaging and visualization compared with a conventional FITC-labeled antibody. The cross-selectivity tests and the dilution tests confirmed that the developed CPE-antibody conjugates have excellent cross-selectivity and also suitable for quantitative cell counting and cell sorting such as fluorescence-activated cell sorting (FACS). Cell viability and proliferation study confirmed that Jurkat and SUDHL-4 showed normal growth and proliferation when they were incubated with CPEs solution, indicating that the CPEs are not cytotoxic. The results demonstrate that as a fluorescent reporting molecule the biocompatible, water-soluble, and emissive CPEs are potentially superior to small molecular dyes and cytotoxic heavy-atom based quantum-dots. The developed CPEs and the convenient direct bioconjugation strategy are readily applicable to other biological molecules.

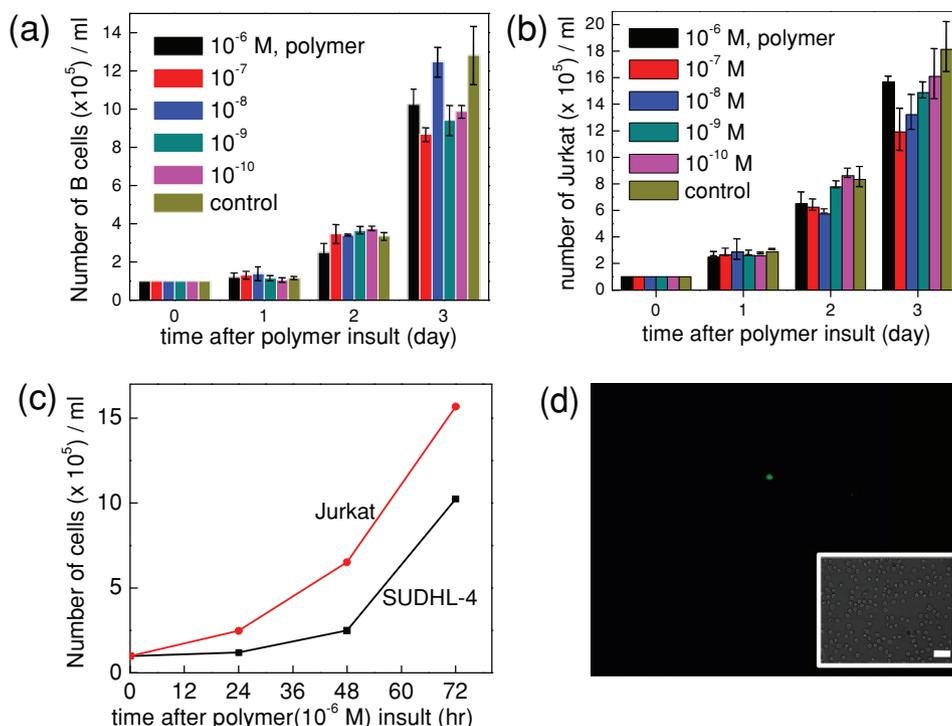


Figure 6. Cell viability and proliferation of (a) SUDHL-4 and (b) Jurkat incubated with different concentrations of PPE1 for 3 days. The concentration of PPE1 in the media was varied from 0.05 to 500 $\mu\text{g}/\text{mL}$ (in $\mu\text{g}/\text{mL}$, 500: Black, 50: red, 5: blue, 0.5: turquoise, 0.05: pink, 0: khaki (positive control)). Trypan blue reagents were added to a small aliquot of the samples, and the number of dead cells and live cells were counted by a hemacytometer in every 24 hours after the incubation with PPE1. (c) A cell proliferation curve of SUDHL-4 (black) and Jurkat (red) upon incubation with the highest concentration of PPE1 (500 $\mu\text{g}/\text{mL}$). (d) A fluorescence microscope image of SUDHL-4 after 1 h from the incubation with PPE1. 100 μl of SUDHL-4 cell media (1 millions cells) was incubated with 10 μl of 3 μM PPE1. The image was obtained after unbound PPE1 was washed off by spinning with fresh cell media (500 \times g, 6 min) several times. (Inset) a DIC image corresponding to the fluorescence image.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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