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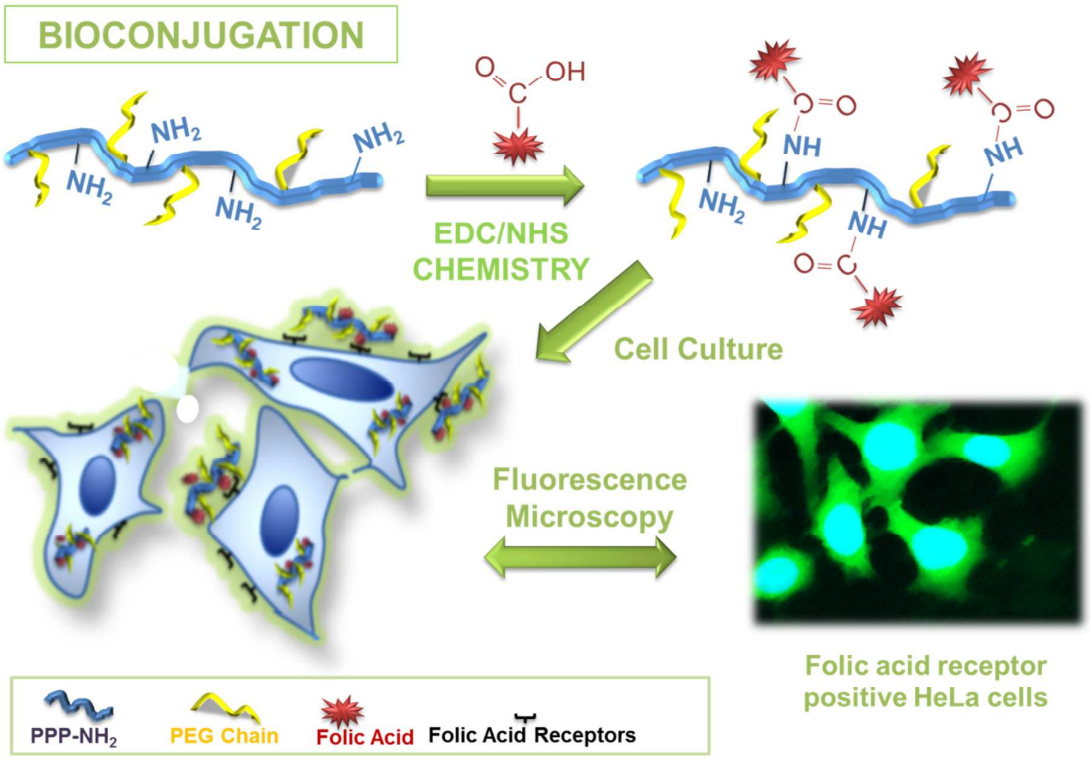
Graphical Abstract



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Journal Name

ARTICLE

Synthesis, Characterization and Targeted Cell Imaging Applications of Poly(p-phenylene)s with amino and poly(ethylene glycol) Substituents

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A novel approach for bioconjugation associated with fluorescent conjugated polymer is demonstrated. For this purpose, a conjugated polymer, poly(p-phenylene) (PPP), with lateral substituents, namely primary amino groups and poly(ethylene glycol) (PEG) chains, as potential building block for polymer bioconjugates was synthesized and characterized. The synthesis was achieved through Suzuki polycondensation reaction in the presence of $Pd(PPh_3)_4$ catalyst by using independently prepared PEG and amino functional dibromo benzenes in conjunction with benzene diboronic acid. For the evaluation of the bioactive PPP labeled with folic acid (FA) as a potential targeted cell imaging probe, HeLa and A549 cancer cells were used. Cytotoxicity assay showed that the polymer was not toxic to the both cells. Additionally, the fluorescence images showed that, depending on the level of the FA receptors on the cell surfaces, the fluorescent intensity in HeLa cells was obviously higher than A549 cells when treated with FA conjugated PPP-NH_2-*g*-PEG polymer. The resulting FA/PPP-NH_2-*g*-PEG conjugate was successfully used as bioconjugate for targeting and specifically imaging of FA receptor positive HeLa human cervices cancer cells.

Introduction

Bio-functionalization of polymers of synthetic origin have played a challenging role over the past decades in the wide range of bioconjugation applications including the covalently incorporation of synthetic polymers with biological materials such as antibodies, peptides, proteins, enzymes, carbohydrates, viruses or cells.[1] Conceptually, this complementary strategies over conjugation of peptides, proteins or enzymes with synthetic polymers to design the versatile molecular biomaterials, had been first considered as essential topic in pharmaceutical chemistry for a few decades.[2-5] In line with recent discoveries of nanotechnology and biotechnology, polymer bioconjugates have gone far the field of biomaterial and gained enormous attraction in material science consisting of artificial enzymes, biometrics, light-harvesting systems, bio-sensors, photonics and nano-electronic devices.[6-8]

To generate a bioconjugation system with synthetic polymer and biomaterial through covalent linkage, the most common method is the carbodiimide-mediated coupling reaction. As cross-linking agents, carbodiimides (EDC or DCC)/N-hydroxysuccinimide (NHS) gives coupling reaction between the primary amine groups in polymer and the carboxyl groups in biomaterial or vice versa positions. Other similar approaches based on chemical modification of primary amine groups to make them appealing different bioconjugation pathways.[9-11] Recent applications related to amino-based systems include, in situ enzymatic photo-patterning for manipulation of cell,[12] amine-modified carbon nanotubes and nonporous carbon in controlled drug releasing,[13-14] non-natural amino acid-mediated protein and polysaccharide conjugation for synthetic biology purposes,[15] bio-based covering material for surface design and biosensor fabrication.[16-17] Amino-rich polymer-based nanoparticles for imaging-guided drug delivery and therapy,[18] biological labelling application of protein by aminated luminescent nanoparticles,[19] polyamine conjugates in systematic *in vivo* short interfering RNA delivery therapeutics,[20] surface initiated living polymerization of N-carboxyanhydride (NCA) to obtain polypeptides by grafting[21-23] are the other typical uses and numerous other studies remain. Although the existence of amine group on polymer provides a unique property and advantage in bio-functionalization process, water-solubility is essential additional requirement in

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Electronic Supplementary Information (ESI) available: [^{1}H-NMR spectra of DBB-MA (Fig. S1), ^{1}H-NMR spectra of PPP-NH_2-*g*-PEG (Fig.S2), Fluorescence excitation and emission spectra of PPP-NH_2-*g*-PEG (Fig.S3), FT-IR spectrum of FA, PPP-NH_2-*g*-PEG and FA/PPP-NH_2-*g*-PEG (Fig.S4)]. See DOI: 10.1039/x0xx00000x

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biological application. Water solubility can be achieved by water soluble supports such as poly(ethylene glycol) (PEG), which comes to the forefront from a highly versatile class of polyethers. PEG was also approved by the US-American Food and Drug Administration for the consuming in human body, exhibiting no antigenicity, immunogenicity, or toxicity.[24-33]

Besides the effective role of PEG in bio-applications, advances in the field of bio-imaging have profoundly impacted the use of water soluble bio-polymers and concerns significantly focused on the fabrication of fluorescence-based organic conjugated polymers relying on π-electron delocalization features.[34] Among the vast number of conjugated polymers, PPP is the most promising polymer in terms of relatively high photoluminescence (PL), electroluminescence (EL) quantum efficiencies and thermos-oxidative stability. PPP can be easily functionalized to form a wide range of its derivatives due to fact that in the synthesis processes, generally the monomer is chosen as phenyl compounds allowing to many chemical modification readily.[35-40] Unfortunately, the main drawback, PPP has, is the insolubility in many solvents, which limits their processability. However, the addition of conformationally appropriate alkyl or water soluble pendants to backbone can lead to obtain soluble PPPs with sufficiently high molecular weight.[41-49]

Nowadays, the goal and the content of the studies in biomedical field are focused on increasing delivery of the variety of drugs, gene therapy agents, and radiopharmaceuticals to the target tissue. A variety of ligands has been examined for the development of efficient targeted imaging, drug delivery and tracking systems.[50] The folate receptor has been proven as a highly selective overexpressed tumor marker in epithelial lineage tumours such as ovarian, colorectal, and breast cancer by the side of healthy tissue.[51] Besides, it is well-known that folic acid (FA) has a high affinity to the folate receptors and is widely used as a targeting ligand for tumor-specific delivery. The inclusion of FA to the targeting system simplifies the internalization of the molecules by the receptor overexpressed cancer cells via receptor-mediated endocytosis.[52]

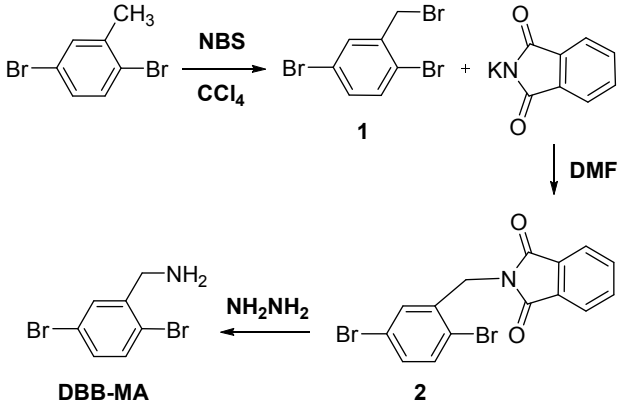
In this study, we describe design and synthesis of fluorescent conjugated polymer possessing amino and PEG side groups (PPP-NH_2-*g*-PEG) suitable for cell-targeting and imaging studies. In this connection, it should be pointed out that there exist several other promising approaches for surface modification or targeting tissues of interest utilizing acid functionalized polymers or anionic polysaccharides-based nanoparticles[ref]. However, the benefits of our system are several folds. In our approach, both amine and hydrophilic PEG functionalities are attached using the same chemistry that facilitates bio-functionality and water solubility, respectively.

The obtained polymers have excellent photophysical properties that can be benefited for the image proposes without any additional fluorophore. Besides, the some problems associated with the nanoparticles are eliminated due to the fully organic and biocompatible polymeric structure.

As will be shown below, the obtained water-soluble polymer was decorated with FA for the targeting and specifically imaging of FA receptor positive HeLa human cervices cancer cells. The FA conjugated PPP-NH_2-*g*-PEG polymer was obtained by a simple bioconjugation method called EDC/NHS chemistry. The resulted conjugates were characterized by several methods such as FTIR and fluorescence spectroscopy. Finally, the cellular internalization of fluorescent-based polymer was visualized by fluorescence microscopy.

Results and discussion

The instruction of bioactive polymer embodying amino and PEG lateral substituents on the same linear conjugated chain needs two different functional monomers in the time that Suzuki polycondensation was chosen as the polymerization technique. Suzuki coupling reaction providing more straightforward access to obtain polymer with changeable sequence ratio between amino functional and PEG monomers was used to adjust the higher contribution of amino monomer than PEG macromonomer to PPP structure. Amino functionality is the crucial part of this study for the utilization of the polymer for bioconjugation. By this association, the preparation of primary amine functional monomer via Gabriel synthesis is based on the transformation of the primary alkyl halides into the primary amines.[53-60] To obtain primary alkyl halide, starting material was chosen as 2,5-dibromotoluene as it can readily react with *N*-bromosuccinimide (NBS) to afford allylic bromination of methyl group of toluene in the presence of peroxide. In the first stage, the reaction between 1 and conjugate base of phtalimide gives the corresponding *N*-alkyl phtalimide. The efficiency of the reaction was increased by using preformed potassium salt of phtalimide instead of phtalimide. Reaction proceeded with a polar protic solvent DMF under homogenous condition and the substitution product methyldibromophenyl phenothiazine (2) was obtained in a higher state of purity and in a shorter period of time. Second stage, named as hydrazinolysis, is the treatment of imide with hydrazine in methanol, results phthalhydrazide and DBB-MA through intramolecular substitution. With the column chromatograph, the amino compound purified and eliminated from the side product of phthalhyrazide (Scheme 1).



Scheme 1. Synthesis of amino functional monomer, DBB-MA.

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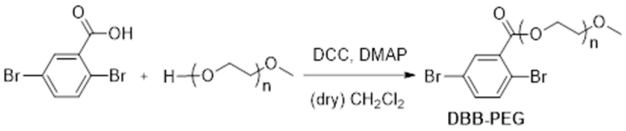
DBB-MA was characterized by ^{1}H-NMR analysis. Singlet peak at 1.59 ppm was assigned to -NH_2 protons. To further confirm the existence of primary amine, hydrogen–deuterium exchange reaction was performed. Notably, after the deuterium exchange with D_2O, this peak disappears. The detailed assignment of the all characteristic peaks is presented in Figure S1 (Supplementary Information).

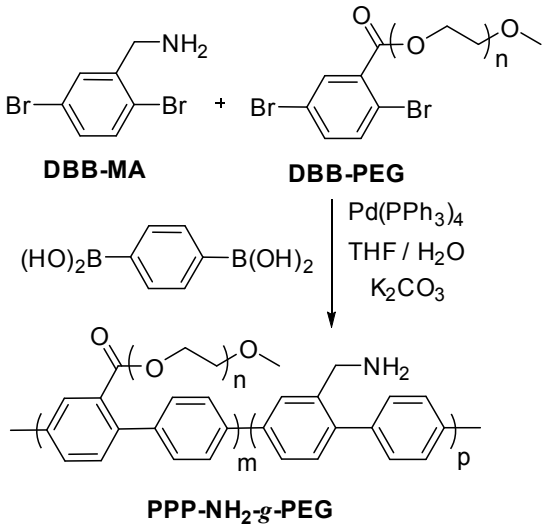
The other dibromo benzene component for the subsequent Suzuki coupling, water soluble macromonomer was obtained by mild Steglich method with the favorable catalytic action of 4-dimethylaminopyridine (DMAP).[61] Esterification reaction between PEG monomethylether and 2,5-dibromobenzoic acid under the standard condition gave the desired product in good yield (Scheme 2). After the elimination of the excess amount of dicyclohexylcarbodiimide (DCC) and unreacted PEG monomethylether by column chromatography, the macromonomer with M_n = 5550 g mol^{-1} and M_w/M_n = 1.24 was obtained.



Scheme 2. Synthesis of water soluble macromonomer, DBB-PEG, via Steglich esterification.

Synthesis of PPP with primary amine groups and PEG side chains.

The combination of PEG and amine functional monomer on the PPP backbone was achieved by Suzuki condensation polymerization using 1,4-benzenediboronic acid as the coupling partner in the presence of palladium catalyst (Scheme 3).



Scheme 3. Synthesis of PPP-NH_2-*g*-PEG via Suzuki condensation polymerization.

Obviously, if desired, the polymer may further be modified by the same reaction through terminal boronic acid or bromobenzene groups.[61-62] Addition of coupling partner makes it possible to arrange the sequence proportion of the components in the conjugated polymer. In our work, we deliberately used the molar ratio of DBB-PEG/DBB-MA as 1/3 to achieve sufficient chain growth since DBB-PEG is relatively less reactive due to the polymeric and hydrophilic nature. Moreover, once adequate hydrophilicity is achieved, the amino functionality is crucial for the subsequent bioconjugation. In the ^{1}H-NMR spectrum, characteristic benzyl amine protons appear at 1.96 ppm as a broad peak which shifts to 4.71 ppm after D_2O exchange (Figure 2S, Supplementary Information).

The M_n and M_w/M_n values of PPP-NH_2-*g*-PEG soluble polymer were found to be 24500 g mol^{-1} and 1.41 as determined by gel permeation chromatography (GPC). This value should be taken as the minimum estimation[63-64] since the polymer has hydrophilic side chain and comb-like structure and the GPC system was calibrated with linear polystyrene standards. Figure 1 shows the GPC traces of the macromonomer and the final conjugated polymer. The trace of the conjugated polymer consists a weak shoulder at high elution volume pertaining to the unreacted PEG macromonomer. This may arise from the difficulty to achieve exact equimolarity particularly when Suzuki coupling components with different molecular weights are used. Due to the similar solubility properties, the separation of the macromonomer by extraction was not possible. However, the presence of small but some macromonomer in the conjugated polymer would not affect the ultimate use in the bioconjugation process, since PEG is known to be a biocompatible polymer.

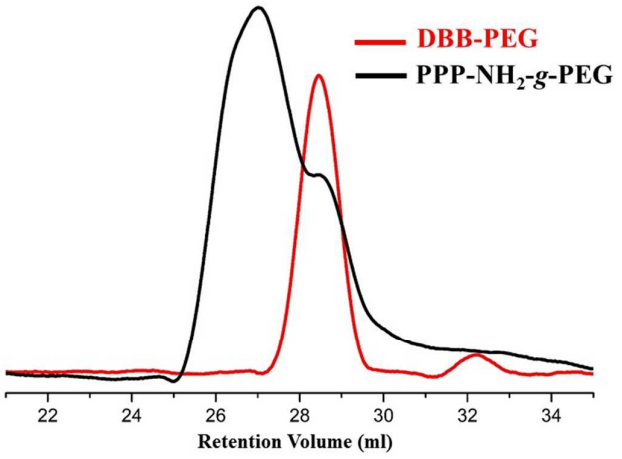


Figure 1. GPC traces of PPP-NH_2-*g*-PEG and DBB-PEG.

UV-Vis absorption spectra of the components of the coupling polymerization and the final polymer are shown in Figure 2. Expected strong absorption band of PPP-NH_2-*g*-PEG was due to the presence of supplementary conjugated phenylene rings in the main chain that can be regarded as the further evidence for the formation of PPP backbone.

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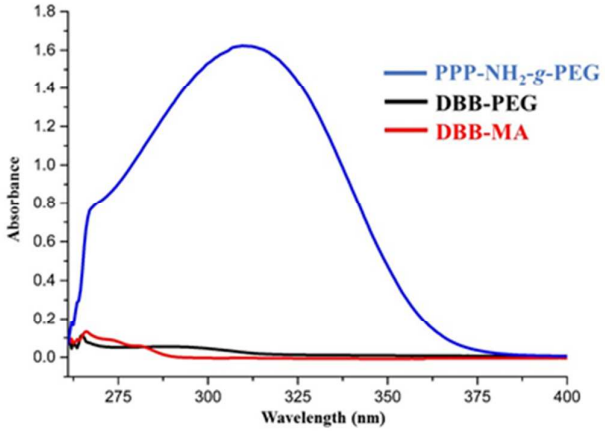


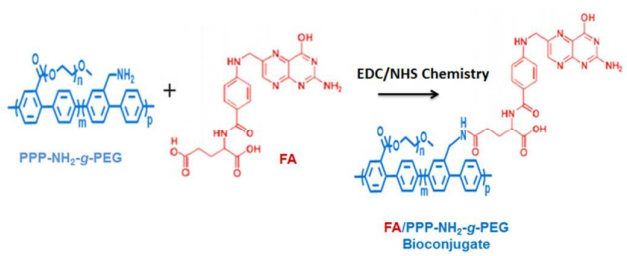
Figure 2. UV absorption spectra of PPP-NH_2-*g*-PEG, DBB-PEG and DBB-MA in DMF, solution (0.1 g/L).

The excitation and fluorescence emission spectra of PPP-NH_2-*g*-PEG in DMF and water were recorded (Figure S3, Supplementary Information). As can be seen, while the excitations in both solvents present similar trend, the corresponding emission in water is slightly blue shifted relative to that in DMF. Moreover, the aggregations that are taking place during the nanoparticles formation through self-assembling of PPP backbones in solution create the red shifted shoulders in asymmetric shape.[65-66]

Normally, as in the case of most conjugated polymer, PPP derivatives have a rigid structure which causes the insolubility problem in common organic solvents. The grafting of relatively long and flexible side chains to the polymer backbone has been a major approach to deal with this problem. On the other hand, the side chains features may change the optical and electronic properties of PPP backbone.[34] In our approach, the solubility is achieved without considerable negative effect on the fluorescence properties of PPP backbone.

Bioconjugation of the FA to PPP-NH_2-*g*-PEG.

The polymer and obtained bioconjugate were characterized by various spectroscopic and microscopic investigations. PPP-NH_2-*g*-PEG was conjugated with FA via a chemical method outlined in Scheme 4.



Scheme 4. Schematic illustration of the bioconjugation.

Fluorescence properties of PPP-NH_2-*g*-PEG polymer before and after bioconjugation were evaluated. As can be seen from the fluorescence spectra illustrated in Figure 3, PPP-NH_2-*g*-PEG has a maximum emission at 410 nm. Upon conjugation, the fluorescence profile of the polymer retained without any major changes. However, conjugation step caused a significant decrease in the fluorescence intensity. However, the remained fluorescence signal is still high enough to use the system for *in vitro* cell imaging experiments, which cannot be realized by conventional fluorophores.

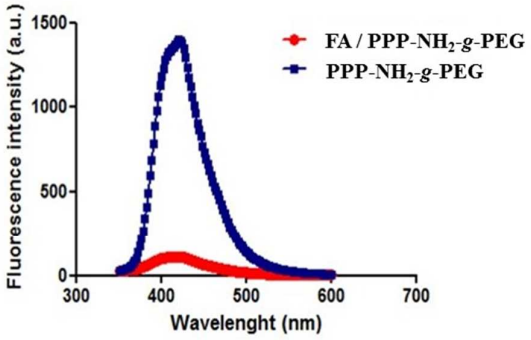


Figure 3. Fluorescence spectra of PPP-NH_2-*g*-PEG and FA/PPP-NH_2-*g*-PEG polymer conjugates.

FTIR analysis was used to confirm the incorporation of the FA in the PPP-NH_2-*g*-PEG, which is shown in Figure S4 (in Supplementary Information). FA is formed from a pteridine ring, *p*-amino benzoic acid and glutamic acid residue. As can be seen from the FTIR analysis of pure FA, the most characteristic peak absorption which was attributed to -COOH and carbonyl groups (C=O) and aromatic C=C structures on both phenyl and pteridine rings was clearly demonstrated itself at the 1684 and 1606 cm^{-1}. After the conjugation of FA with PPP-NH_2-*g*-PEG (which will be then called as FA/PPP-NH_2-*g*-PEG), the absorption peaks at 1684 and 1611 cm^{-1} may be belong to the C=O stretching which is related to amide-I bond. Furthermore, the absorption band between 3300 and 3600 may have belonged to hydroxyl stretching bands that comes from FA.

Cell culture

Cytotoxicity assay. Cytotoxic effects of PPP-NH2-g-PEG and FA/PPP-NH2-g-PEG polymer at different concentrations were examined by MTT assay on A549 and HeLa cells. As demonstrated in the Figure 5, the MTT assay results for HeLa and A549 cells, toxicity of the FA/PPP-NH_2-*g*-PEG polymer was slightly higher than non-conjugated polymer on HeLa cell lines, while both of them showed similar toxicity on A549 cell lines. The differences between toxicity of FA conjugated and non-conjugated polymer may depend on the cellular uptake of the samples. Recently, the expression levels of FA receptors on the surfaces of both cell lines were investigated by using both genomic and proteomic approaches, PCR and flow cytometry analysis, and were found to be overexpressed on surface of HeLa cells compared to A549 cell lines.[67] It is attributed that

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the increase in cytotoxicity was caused by enhanced cellular uptake of FA conjugated polymer in FA receptor positive HeLa cells via receptor-mediated endocytosis.

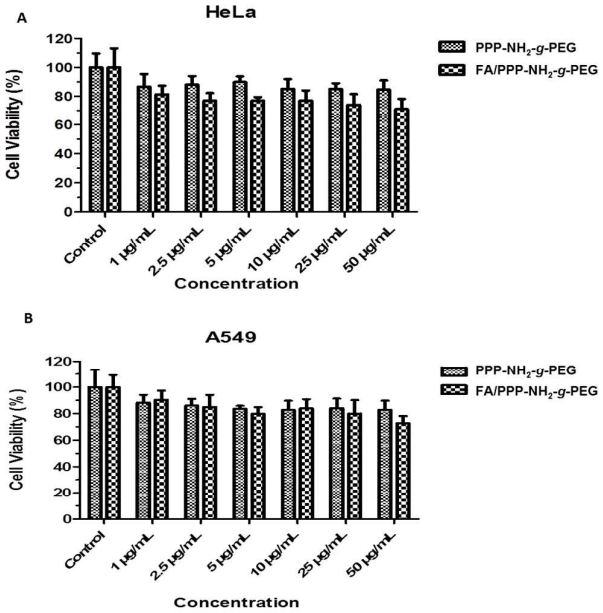

Figure 5. The effect of PPP-NH_2-*g*-PEG and FA/PPP-NH_2-*g*-PEG polymer on cell viability of HeLa cells **(A)** and A549 cells **(B)**. Values are the mean ± standard deviation (n = 6).

Fluorescence microscope images. FA receptor positive cells have no pores or channels for the diffusion of the polymeric materials into the cells. In order to achieve more efficient therapy and imaging studies, it is important to use of membrane permeable agents.[68] FA functionalized materials constitute a considerable target for tumor specific delivery of variety of drugs, gene therapy agents, and radiopharmaceuticals for FA receptor rich cell lines such as HeLa.[69] For the investigation of FA/PPP-NH_2-*g*-PEG conjugate as a potential targeted cell imaging probe, fluorescence microscope images of the HeLa and A549 cells were taken after incubation of the samples. The fluorescence images of HeLa and A549 cells treated with the FA/PPP-NH_2-*g*-PEG polymer and non-conjugated polymer were given in Figure 6. According to fluorescence microscope images of cells, the visual fluorescence signal is increased in HeLa cells after being treated with FA/PPP-NH_2-*g*-PEG polymer in comparison with non-conjugated polymer. As shown in Figure 6-A, functionalization of the PPP-NH_2-*g*-PEG polymer with FA increased the cellular uptake in HeLa cells through FA receptors on cell surfaces while the non-conjugated polymer was not endocytosed (Figure 6-B). In contrast to this, for FA receptor poor A549 cells, no differences were observed between the fluorescence signal both of FA conjugated and non-conjugated polymer samples (Figure 6A-B). As it can be seen from the fluorescence images, the fluorescent intensity in HeLa cells was obviously higher than A549 cells, depending on the uptake selectivity of FA conjugated polymer. Also, fluorescence intensity of the FA/PPP-NH_2-*g*-PEG polymer in FA pre-treated HeLa cells significantly decreased because of the

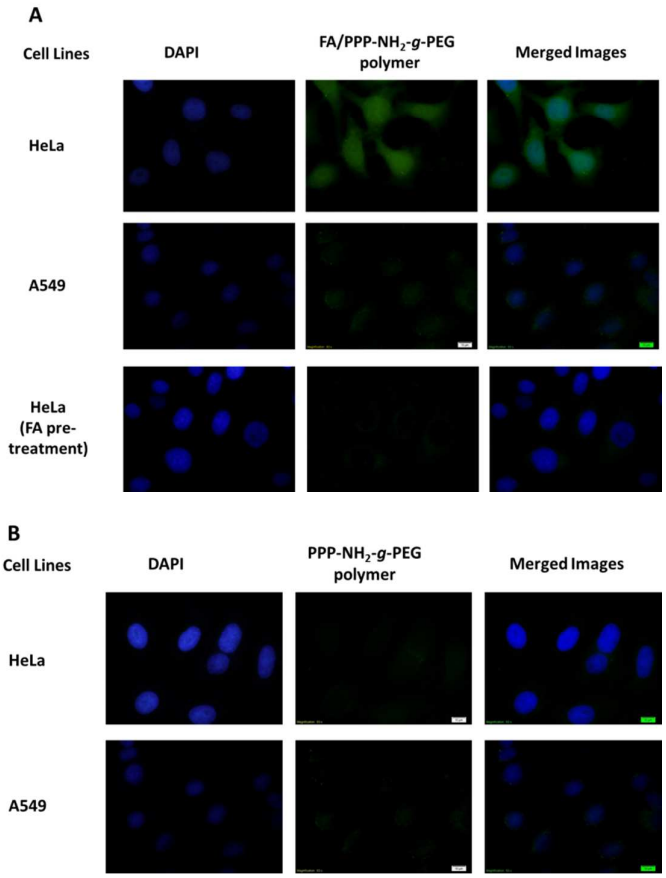

Figure 6. Fluorescence microscopy imaging of HeLa and A549 **c**ells. Cells were treated with FA/PPP-NH_2-*g*-PEG polymer directly and after being pre-treated with free FA **(A)**. Cells were treated with non-conjugated PPP-NH_2-*g*-PEG polymer **(B)** for 2 h at 37°C, overlap of two images, control nuclei staining with DAPI.

saturation of FA receptors with free FA. Based on these results it can be said that the internalizing of the polymer by the HeLa cells is dependent on endocytosis via FA receptors. Besides, all of this data is coherent with the cytotoxicity assay results.

Experimantal

Materials

Tetrakis(triphenylphosphine)palladium(0) ($Pd(PPh_3)_4$), 2,5-Dibromo-benzoic acid, 1,4-benzene-diboronic acid, *N*-Bromosuccinimide (NBS), 4-(*N,N'*-dimethyl) amino pyridine (DMAP), dicyclohexylcarbodiimide (DCC), poly(ethylene glycol)

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mono methyl ether (PEG_{2000}), Phthalimide potassium salt, hydrazine monohydrate (98%) and Benzoyl peroxide, Folic acid, *N*-(3-dimethylaminopropyl)-*N*'-ethylcarbodiimide hydrochloride, Sigma *N*-Hydroxysuccinimide, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, sodium dodecyl sulfate, Diamino-2-phenylindol were purchased from Sigma-Aldrich and used without any further purification. All solvents were purified and dried. Water was purified in a Milli-Q plus System (Milli-pore).

Measurements

^{1}H-NMR spectra were performed with an Agilent VNMRS 500 MHz, and chemical shifts were recorded in ppm units using tetramethylsilane as an internal standard. UV/vis absorbance measurements were recorded on a Shimadzu UV-1601 spectrometer. Fluorescence measurements were performed with a model LS-50 spectrometer from Perkin Elmer at room temperature. Gel-permeation chromatography (GPC) measurements were carried out in Viscotek GPCmax auto sampler system. Instrument was equipped with a pump (GPCmax, Viscotek Corp., Houston, TX), light-scattering detector (λ_0 = 670 nm, Model 270 dual detector, Viscotek Corp.) consisting of two scattering angles: 7° and 90° and the refractive (RI) index detector (VE 3580, Viscotek Corp.). Both detectors were calibrated with PS standards in the narrow molecular weight distribution. Three ViscoGEL GPC columns (G2000HHR, G3000HHR and G4000HHR) empoyed with THF in the 1.0 mL min^{-1} flow rate at 30^{o}C. All data were analyzed using Viscotek OmniSEC Omni-01 software.

Synthesis of 1,4-dibromo-2-(bromomethyl)benzene (1). 2,5-Dibromotoluene (2.55 mL, 18.5 mmol), NBS (6.2 g, 24.8 mmol) and 0.1 g of benzoyl peroxide were added to dry 40 mL of CCl_4 under nitrogen atmosphere. The solution was refluxed with condenser under nitrogen and 200 W light for 4 h. Heating started from 65 °C and temperature was increased by 10 °C per hour and in the last one h temperature was kept at 95°C. After that, the precipitate was filtered and washed with a supplementary amount of CCl_4 and finally with a small quantity of CH_2Cl_2. The organic layer was washed with water several times, and then dried over $NaSO_4$. The solvent was evaporated in *vacuo*. Then the remaining crude material was purified by flash column chromatography (SiO_2, Et_2O) and recrystallized in petroleum ether to yield (1) (3.04 g, 50%, white crystal). ^{1}H-NMR ($CDCl_3$, 500 MHz): δ 4.54 (s, 2H), 7.30 (dd, *J* = 8.5, 2.4 Hz, 1H), 7.45 (d, *J* = 8.5 Hz, 1H), 7.60 (d, *J* = 2.3 Hz, 1H).

Synthesis of 2-(2,5-dibromobenzyl)isoindoline-1,3-dione (2). To the solution of **1** (2.9 g, 8.8 mmol) in 10 mL dry DMF, potassium phthalimide (2.45 g, 13.22 mmol) were added. After stirring for 12 h at 160 °C in a schlenck tube under nitrogen atmosphere, the reaction mixture was poured into water. The precipitate was washed with water, purified by flash column chromatography (SiO_2, CH_2Cl_2), precipitated in ethanol and dried under vacuum to yield (2) (2.56 g, 74%, white solid). ^{1}H-NMR ($CDCl_3$, 500 MHz): δ 4.94 (s, 2H), 7.26 (dd, *J* = 5.7, 2.5 Hz, 1H), 7.28 (d, *J* = 2.3 Hz, 1H), 7.45 (d, *J* = 8.3 Hz, 1H), 7.79 (dd, *J* = 5.5, 3.0 Hz, 2H), 7.92 (dd, *J* = 5.5, 3.1 Hz, 2H).

Synthesis of (2,5-dibromophenyl)methanamine (DBB-MA). The solution of **2** (0.381 g, 0.95 mmol) in 25 mL absolute ethanol with 3.0 mL hydrazine were stirred at 90 °C for 5 h. After allowing cooling to room temperature, the mixture was extracted with CH_2Cl_2 and 10% $NaHCO_3$. The organic layer was dried over Na_2SO_4 and reduced in *vacuo*. The residue was purified by column chromatography (Al_2O_3 (neutral), THF) to yield (DBB-MA) (145 mg, 57%, yellow oil).). ^{1}H-NMR ($CDCl_3$, 500 MHz): δ 1.59 (br, 2H), 3.87 (s, 2H), 7.23 (dd, *J* = 8.4, 2.4 Hz, 1H), 7.38 (d, *J* = 8.4 Hz, 1H), 7.54 (d, *J* = 2.4 Hz, 1H).

Synthesis of PEG macromonomer (DBB-PEG). 2,5-Dibromo-benzoic acid (4.0 g, 14.3 mmol), DMAP (174.7 mg, 1.43 mmol), Me-PEG_{2000}-OH (25.74 g, 12.87 mmol) were dissolved in 80 ml of dry CH_2Cl_2 under nitrogen atmosphere. To this solution was added DCC (3.246 g, 15.7 mmol) in 20 mL dry CH_2Cl_2 drop-wise under nitrogen. Then, the reaction mixture was stirred for 5 days at room temperature. After that, the mixture was filtered and washed with 250 mL of CH_2Cl_2. The organic phase was quenched with 10% $NaHCO_3$ and brine, and then dried over Na_2SO_4. The solution was concentrated and passed through a silica-gel column using CH_2Cl_2 as eluent. After removal of solvent in *vacuo*, the polymer was precipitated in cold diethyl ether to yield 26 g (95%). ^{1}H-NMR ($CDCl_3$, 500 MHz): δ 3.85 – 3.46 (broad), 3.38 (broad), 7.45 (dd, *J* = 8.5, 2.4 Hz, 1H), 7.52 (d, *J* = 8.5 Hz, 1H), 7.93 (d, *J* = 2.4 Hz, 1H).

Synthesis of PPP conjugated polymer (PPP-NH_2-*g*-PEG). The reaction solution was prepared with 30 mL THF and 20 mL of 2.0 M aqueous K_2CO_3 solution under nitrogen. Prior use, this solution was degassed by bubbling nitrogen over a period of 30 min. In the 10 mL of the reaction solution, DBB-MA (0.8 g, 3.0 mmol), DBB-PEG (2.3 g), benzene-1,4-diboronic acid (0.663 g, 4.0 mmol), $Pd(PPh_3)_4$) (0.092 g, 0.08 mmol) was dissolved under nitrogen and stirred at 70^{o}C for 4 days in the Schlenk tube under vacuum. After stirring, the reaction mixture extracted with CH_2Cl_2 and small amount of water. Organic phase was concentrated and precipitated in excess diethyl ether to yield 1.85 g, white powder. ^{1}H-NMR ($CDCl_3$, 500 MHz): δ 2.06 (broad), 3.38 (broad), 3.45-3.8 (broad), 7.92-8.20 (broad), 7.6-7.9 (broad), 7.2-7.6 (broad).

Bioconjugation of FA to PPP-NH_2-*g*-PEG Polymer. FA/PPP-NH_2-*g*-PEG polymer conjugates were prepared by conventional EDC/NHS chemistry. FA was conjugated to the polymer through the reaction between amino group of the polymer and carboxyl group of FA. Briefly, FA (25 µg/mL) dissolved in PBS (pH 7.4) reacted with EDC (0.5 M, in 25 mM, pH 5.5 MES buffer) and NHS (0.125 M, in 25 mM, pH 5.5 MES buffer) for the activation of the carboxyl group of FA. Then, the activated FA was reacted with PPP-NH_2-*g*-PEG polymer containing amino group for 4 h at room temperature. Finally, the conjugates were washed with PBS three times by using 10 kDa membrane filter (Sartorius Stedim Biotech).

Characterization of the FA/PPP-NH_2-*g*-PEG conjugate. The structure of polymer can be easily modified with FA due to the presence of functional amino groups in the structure. To verify the successful conjugation of polymer and FA, the fluorescence intensity of polymer was monitored with microplate reader (Thermo Scientific, Varioskan Flash Multimode Reader, USA)

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step by step. Also, the conjugation was confirmed FTIR analyses. FT-IR spectra were recorded on a PerkinElmer FT-IR Spectrum One spectrometer.

Cell culture. A549 (human lung cancer) and HeLa (human cervices cancer) cell lines were obtained from ATCC. Cells were cultured on DMEM containing 10% FCS and 1.0% penicillin/streptomycin and were incubated at 37°C in a 5.0% CO_2 and 95% air humidified atmosphere.

Cytotoxicity assay. Determination of the dose dependent cytotoxicity of the PPP-NH_2-*g*-PEG polymer and prepared conjugates was carried out via 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay.[67] Cells were removed from flask to 96-well-tissue plates and were cultivated in the wells until reaching confluence. After completion of cultivation, the medium was removed and the cells were washed with PBS. Following the washing of wells, the cells were treated with the PPP-NH_2-*g*-PEG and FA/PPP-NH_2-*g*-PEG polymers at 50 µg/mL, 25 µg/mL, 10 µg/mL, 5.0 µg/mL, 1.0 µg/mL concentrations, for 24 h. Then the samples were removed completely by washing with PBS and 10% MTT solution was added to the wells. Afterwards, cells were incubated with MTT for 4 h and 100 µL SDS (1.0 g SDS in 10 mL 0.01 M HCl) was added per well for dissolving formazan which was occurred inside the cells as a result of MTT treatment. After 24 h of incubation, the optical densities of each well were measured by using a spectrophotometric plate reader (Bio-Tek Instruments, Winooski, VT, USA).

Cell imaging via fluorescence microscopy. For the purpose of examination of the interactions between the targeted and non-targeted fluorescence probes with both FA receptor negative and positive cell lines, A549 and HeLa cells were imaged via fluorescence microscope (Olympus BX53F) equipped with a CCD camera(Olympus DP72) by utilizing the fluorescence properties of the polymer. In order to obtain cell images, FA conjugated polymer and non-conjugated polymer (10 $\mu g/mL^{-1}$ as the polymer amount) were diluted with medium 1:1 and were added to cells cultivated on a chamber slide for 2 days. The cells were incubated for 2 h at 37°C and were washed twice with PBS. At the end of the incubation time, the cells were stained with DAPI. Additionally, for the saturation of the FA receptors with free FA prior to treatment with FA/PPP-NH_2-*g*-PEG, first FA (500 $\mu g/mL^{-1}$) was diluted with medium 1:1 and added to HeLa cells cultivated on a chamber slide for 2 days. The cells were incubated for 1 h at 37°C and washed twice with PBS. Then, FA/PPP-NH_2-*g*-PEG polymer was added to the chamber slide and was incubated with cells for 2 h at 37°C. Afterwards, the cells were washed twice with PBS. In the final step, DAPI was added for the nuclei staining of intact cells. The treated cells were visualized via the fluorescence of the polymer and DAPI by using two filters (green emission; 515-550 nm, blue emission; 435-485 nm) under the fluorescence microscope.

Conclusion

The synthesis of PPP structure bearing PEG and amine group with high water solubility and stable fluorescence feature may lead new pathways in the bioconjugation field. The novel targeted fluorescence bio-probe was successfully synthesized and applied to both HeLa and A-549 cells for examination of differences of the cellular affinity between targeted and non-targeted samples. All of our results demonstrated that the florescent property of synthesized PPP-NH_2-*g*-PEG polymer can be appropriate for effective cell imaging application. In addition to that, the results showed that PPP-NH_2-*g*-PEG polymer cannot diffuse into the HeLa and A549 cells when it is in non-conjugated form. The characteristic attributes of the polymer has shown to be a great model for the targeting systems. As a final addition, it was demonstrated that the PPP-NH_2-*g*-PEG polymer is an excellent candidate for the targeting systems when it is appropriately functionalized.

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