Article

Chinese Science Bulletin

SPECIAL ISSUE: Nano-Biomedical Optoelectronic Materials and Devices

July 2013 Vol.58 No.21: 2570–2575
doi: 10.1007/s11434-013-5796-0

Star-shaped conjugated oligoelectrolyte for bioimaging in living cells

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Received September 6, 2012; accepted January 29, 2013; published online May 3, 2013

A new star-shaped oligoelectrolyte (TEFCOONa) with triphenylamine as the core, acetylene as linkage and anionic fluorenes as arms was obtained and used for direct imaging in living PANC-1 cells. Because of the hydrophobic conjugated groups of the oligoelectrolyte, TEFCOONa can form nanospheres with an average diameter of ~75 nm in 10 mmol/L PBS. These nanospheres possess a relatively high absolute quantum yield (16.5% in PBS), low cytotoxicity and can penetrate into the nucleus through the cytoplasm, which is essential for living cellular imaging. Collectively, these results validate our rational design of conjugated oligoelectrolyte and even hyper branched polymers-copolyelectrolyte as effective nanovectors for bioimaging and other clinical applications.

star-shaped, oligoelectrolyte, absolute quantum yield, nanospheres, bioimaging

Citation: Song W L, Jiang R C, Yuan Y, et al. Star-shaped conjugated oligoelectrolyte for bioimaging in living cells. Chin Sci Bull, 2013, 58: 2570–2575, doi: 10.1007/s11434-013-5796-0

Over the past decades, conjugated polyelectrolytes (CPEs) have been widely used in sensing and cell imaging due to their intriguing optoelectronic and biocompatible properties [1–6]. Structurally, conjugated polyelectrolytes (CPEs) are fluorescent macromolecules with electron-delocalized backbone and water-soluble side chains which determine the main optical properties and capability to dissolve in water for their further biological applications respectively. So far, plenty of CPEs have been obtained through palladium-catalyzed coupling reactions (Suzuki, Heck, Snogashira), such as polyfluorene (PF), poly(*p*-phenyleneethynylene) (PPE), polydiacetylene (PDA), poly(thiophene) (PT) [7–9] and so on. These materials owning relatively high photoluminescent (PL) quantum yield (QY) and good water-solubility as depicted have formed an excellent basis for chemical and biological sensors. But their applications in cell imaging are seldom reported, possibly because the linear geometries affect the cellular uptake process [10]. Therefore it is necessary to exploit CPEs with suitable geometries for cell imaging.

On that basis, hyperbranched conjugated macromolecules with three-dimensional (3-D) architectures and water-soluble groups were designed and synthesized for a variety of applications especially for cell imaging. Their three-dimensional architectures usually contain three parts: photosynthetic centers giving them advantages for light harvesting, one or more branches improving the π-electron delocalization [11,12], and terminal groups [13]. All of these realize the electron and energy effective transfer through the whole molecular, which is facilitated to light harvesting.

Besides, we can regulate their size by changing groups. Unfortunately, so far water-soluble hyperbranched conjugated polymers are rarely developed, although their organic-soluble counterparts such as three or six-armed polymers [14,15] and so on, have been explored for use in organic

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electronics in many literatures [16]. Notably, Wang et al. [12] and Li et al. [17] have synthesized a series of water-soluble hyperbranched polymers such as star-shaped glycosylated conjugated, hyperbranched conjugated polyelectrolyte and so on for cell imaging recently. Generally, the frames of their polyelectrolytes containing triphenylamine, fluorene, benzene and other groups, were linked by Suzuki reaction or Oralkyne polycyclotrimerization. Figure 1 shows their chemical structures clearly. These materials form different sizes of nanospheres in buffer solution easily. Advantages of the nanospheres: facilitating cellular uptake; low cytotoxicity and possessing high quantum yield, indicate that these polymers are ideal candidates for biological applications. But considering varied clinical applications, it is necessary to exploit a new type of hyperbranched conjugated polymers.

In this contribution, we designed and synthesized a new 3-D star-shaped oligoelectrolyte (TEFCOONa) through the sonogashira reaction firstly. The molecular was designed based on a triphenylamine core and anionic fluorenes, which ensure its strong fluorescence and good water solubility. Dissolved into the buffer solution, the oligoelectrolyte self-assemble to nanospheres and that is good for cytophagy. Then we use RAW 264.7 cells to study the cytophagy. PANC-1 (Human pancreatic carcinoma, epithelial-like cell line) cells were used for cell imaging.

1 Experimental

NMR spectra were recorded on a Bruker Ultra Shield Plus 400 MHz NMR (^{1}H: 400 MHz, ^{13}C: 100 MHz). Mass spectra were obtained on a Bruker Daltonics matrix-assisted laser desorption/ionization time-of-flight mass spectrometer (MALDI-TOF-MASS). The UV-visible absorption spectra were recorded on a Shimadzu UV-3600 UV-VIS- NIR spectrophotometer. Photoluminescent spectra were measured on a RF-5301PC spectrofluorophotometer. Edinburgh instruments F900 was used to obtain the absolute quantum yield. TEM images were obtained from HT7700. BioTek PowerWave XS2 was used to study cell viability with different concentrations of the material. Confocal laser scanning microscopy (CLSM) images of the sample were recorded on Olympus, FV1000.

The 2-bromo-9,9-bis(3′-tert-butylpropanoate) fluorine (**1**) were synthesized according to the previous publication [18]. All chemical reagents used were purchased from Sigma-Aldrich, J&K, and Alfa and were used as-received. Other organic solvents were used without further purification except DMF.

1.1 Synthesis and characterizations

Synthesis of 2-bromo-9,9-bis(3′-tert-butylpropanoate) fluorine (**2**). A sample (5.01 g, 10 mmol) of **1**, (0.35 g, 0.5 mmol) of $PdCl_2(PPh_3)_2$ and (0.0952 g, 0.5 mmol) of CuI were dissolved in 40 mL of diisopropylamine. (2.156 g, 22 mmol) of trimethylsilyl acetylene was added into the vigorously stirred solution slowly at room temperature under nitrogen protection. After the addition was finished, the mixture was stirred at room temperature for 0.5 h. The mixture was warmed to reflux temperature under constant stirring for 12 h. After the solvent was evaporated under reduced pressure, the residue was poured into 100 mL of water and extracted with chloroform three times. The combined organic layer was washed with water twice and brine once and dried over $MgSO_4$. The crude material was purified by silica gel column chromatography, using petroleum as an eluent, to give the product 1 as a slight yellow solid (4.422 g, 96%): ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.71 – 7.59 (m, 2H),

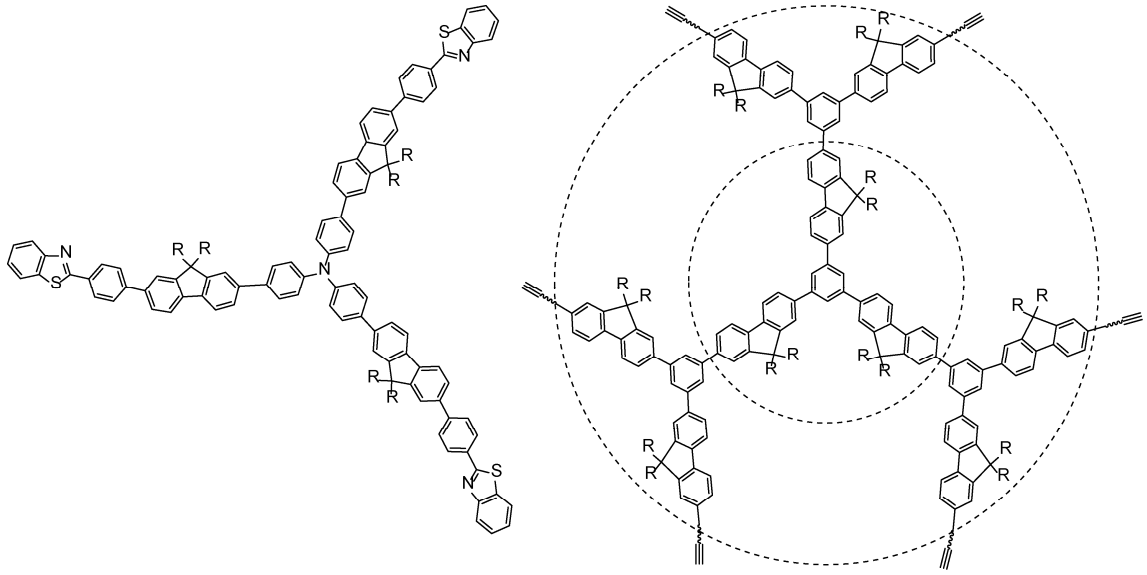

Figure 1 Chemical structures of branched polyelectrolytes (Copyright 2009 and 2011 American Chemical Society).

7.48 (t, J =3.7 Hz, 2H), 7.41 – 7.29 (m, 3H), 2.51 – 2.20 (m, 4H), 1.63 – 1.34 (m, 4H), 1.31 (s, 18H), 0.28 (s, 40H). ^{13}C NMR ($CDCl_3$, ppm): δ 172.66, 148.47, 148.06, 141.56, 140.37, 131.77, 128.19, 127.66, 126.48, 123.10, 121.85, 120.35, 119.82, 105.69, 94.66, 80.17, 77.34, 53.47, 34.56, 29.90, 28.00. FT-IR (KBr pellet, cm^{-1}): 744.52, 846.75, 1151.50, 1203.58, 1251.80, 1286.52, 1369.46, 1390.68, 1415.75, 1450.47, 1728.22, 2927.94, 2976.16, 3367.71.

Synthesis of 2-ethynyl-9,9′-bis(3′-tert-butylpropanoate) fluorine (**3**). In a 250 mL flask, **2** (2.59 g, 5 mmol) was dissolved in a mixture of K_2CO_3 (2 g in 10 mL of water) and 50 mL of methanol and 50 mL of THF, and stirred at room temperature for 6 h. After evaporation of the solvent, the crude product was recrystallized with petroleum/ethyl acetate (1:4, v/v) to afford light yellow crystals (2.12 g, yield 92%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.72 – 7.62 (m, 1H), 7.56 (d, J = 8.0 Hz, 1H),7.53 – 7.46 (m, 2H), 7.42 – 7.31 (m, 3 H), 3.16 (s, 1H), 2.41 – 2.23 (m, 4H), 1.52 – 1.39 (m, 4H), 1.31 (s, 18H). ^{13}C NMR ($CDCl_3$, ppm): δ 172.60, 148.45, 148.16, 141.85, 140.28, 131.85, 128.29, 127.70, 126.72, 123.14, 120.84, 120.39, 119.91, 84.24, 80.19, 77.57, 53.50, 34.54, 29.93, 28.00. FT-IR (KBr pellet, cm^{-1}): 746.95, 857.39, 1150.441, 1287.2, 1370.88, 1724.9, 2929.74, 2977.74, 3243.87.

Synthesis of 4,4′,4″-tris(4-(9,9′-bis(3′-tert-butyl propanoate)fluorenyl-2-)ethynyl) phenylamine (TEFCOOBu) Tris-(4-iodo-phenyl)-amine (311 mg, 0.5 mmol) and **2** (921.2 mg, 2 mmol), $Pd(PPh_3)_4$ (40 mg, 0.035 mmol), (7.2 mg, 0.035 mmol) of CuI , DMF (5 mL) and triethylamine（TEA）(5 mL) were mixed in a 25 mL two-neck flask. After degassing, the mixture was heated at 90°C with vigorous stirring for 48 h. After the mixture was cooled to room temperature and removed the solvent, the crude material was purified by silica gel column chromatography, using petroleum/ethyl acetate (1:6, v/v) as an eluent, to give the product TEFCOOBu as a slight yellow need-like crystal (210 mg, 84% yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.68 (t, J = 6.8 Hz, 5H), 7.51 (dd, J = 18.3, 7.6 Hz, 12H), 7.42 – 7.32 (m, 9H), 7.13 (d, J = 8.6 Hz, 6H), 2.36 (t, J = 8.3 Hz, 12H), 1.52 – 1.45 (m, 12H), 1.30 (s, 54H).^{13}C NMR ($CDCl_3$, ppm): δ 172.70, 148.44, 148.23, 146.72, 141.20, 140.49, 132.82, 131.23, 128.11, 127.68, 126.05, 124.12, 123.12, 122.25, 120.28, 119.97, 117.92, 89.98, 89.90, 80.19, 77.35, 53.50, 38.75, 34.62, 31.60, 29.97, 28.01, 22.67, 22.63, 14.14. MS FT-IR (KBr pellet, cm^{-1}): 742.59, 831.32, 945.12, 1147.65, 1269.16, 1284.59, 1319.31, 1388.75, 1454.33, 1506.41, 1595.13, 1728.22, 2339.65, 2360.87, 2854.65, 2926.01, 2972.31. MALDI-TOF-MASS: m/z 1578.55.

Synthesis of 4,4′,4″-tris(4-(9,9′-bis(3′-tert-propanoate sodium) fluorenyl-2-)ethynyl)phenylamine (TEF-COONa) TEFCOOBu (200 mg) was dissolved in dichloromethane (20 mL) in a 50 mL flask, then trifluoroacetic acid (5 mL) was added into the flask, and the mixture was stirred overnight at room temperature. After removal of the solvent, the yellow-green residue was treated with Na_2CO_3 aqueous solution (0.05 mol/L, 20 mL) at room temperature for 4 h. The polymer was purified through dialysis against distilled water for 3 d. The solution was freeze-dried to give TEF-COONa (65 mg, 76% yield) as yellow powder. ^{1}H NMR (400 MHz, CD_3OD): δ 7.81 (d, 4H), 7.67 (s, 5H), 7.52 (s, 2H), 7.43–7.35 (m, 9H), 7.27 (s, 5H), 6.99 (dd, J = 15.6, 8.0 Hz, 8H), 2.32 (m, 12H), 1.36 (m, 12H). FT-IR (KBr pellet, cm^{-1}): 3410.15(br), 2956.87, 2920.23, 1276.87, 2852.72, 1658.78, 1658.78, 1591.27, 1568.13, 1548.84, 1506.41, 1446.61, 1386.82, 1317.38, 1172.72, 835.18, 740.67.

1.2 Results and discussions

The synthetic route of the oligomers is shown in Figure 2. Compound **1** was prepared in 71% yield by direct alkylation of 2-dibromofluorene with tertbutyl acrylate in a toluene/aqueous KOH mixture, which was followed by purification using silica column chromatography. **2** was synthesized under Sonogashira reaction conditions in the presence of $PdCl_2(PPh_3)_2$, CuI, (trimethylsilyl)acetylene using dry diisopropylamine as the solvent. The NMR spectroscopy, MS, FT-IR spectroscopy results show that the products were obtained in the correct structures with high purity. Oligomerization between TIPA and **3** was conducted in a mixture solution of DMF and TEA under nitrogen atmosphere for 24 h to yield TEFCOOBu. The ^{1}H NMR spectrum of TEFCOOBu in $CDCl_3$ has shown a chemical shift of 1.30 ppm, which corresponds to the protons for the $-C(CH_3)_3$ group, indicating the existence of carboxylic ester groups. TEFCOOBu is soluble in organic solvents. TEFCOONa was produced by hydrolysis of TEFCOOBu in the presence of CF_3COOH and dichloromethane, which was followed by reaction with 0.1 mol/L Na_2CO_3 overnight at room temperature. It was obtained in 76% yield after purification through dialysis (cutoff molecular weight 500) against DI water for 3 d to remove salt and small molecular weight fractions. According to the ^{1}H NMR spectrum of TEF-COONa, the chemical shift at 1.30 ppm disappeared, which indicated the complete conversion of $-COOC(CH_3)_3$ to –COONa. Different from TEFCOOBu, TEFCOONa is soluble in water and methanol.

2 Optical properties and self-assemble behaviors

The UV-Vis and PL spectra of TEF-COONa in water are shown in Figure 3. The oligomer has an absorption maximum of 330 and 376 nm and emits green-blue fluorescence with a main peak at 446 nm. These good optical properties are directly related to its geometrical structure, where the triphenylamine are chosen as the core because of its electron-rich and unique “propeller” type of structure, modified fluorenes are chosen as branches to extend the con-jugation length through the 3-D structure. All of these groups were

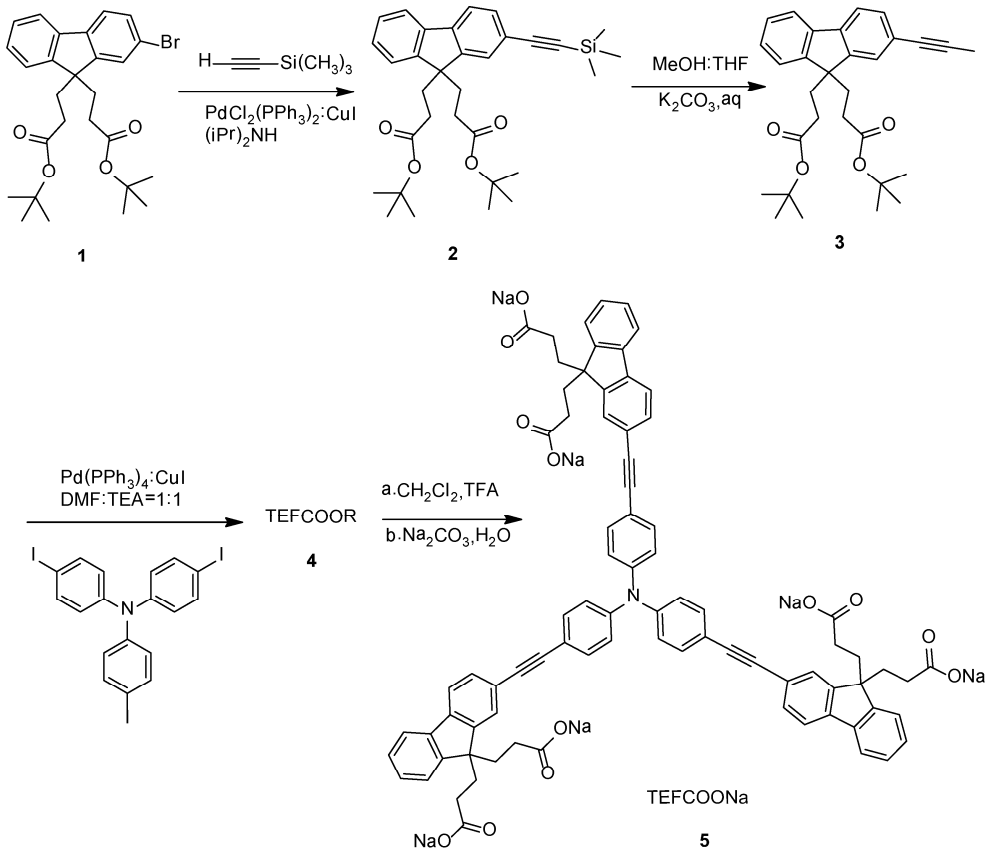

Figure 2 Synthetic route for oligomers **4** and **5**.

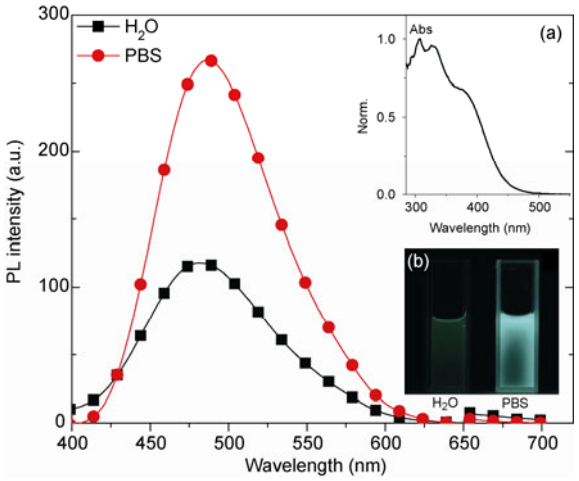

Figure 3 PL spectra (λ_{ex} = 330 nm) of TEFCOONa (50 μg/mL) in water (black) and in 10 mmol/L PBS (red). (a) UV-Vis absorption spectrum of TEFCOONa in water; (b) the emission photos (λ_{ex} = 365 nm) observed of TEFCOONa in water and 10 mmol/L PBS.

linked by acetylene leading to effective π-electron delocalization and high fluorescence quantum yield in water. Furthermore, the unique star-shaped structure can avoid π-π stacking to some extent, which is of benefit for the properties.

The effect of the buffer solution on the optical properties of the oligomer was investigated. The PL spectra of 50 μg/mL TEFCOONa in 10 mmol PBS buffer (10 mmol/L, pH ~7.4) are shown in Figure 3. The maximum emission wavelength red shifts (about 7 nm) in buffer solution is compared with that in water. And fluorescence intensity increases notably in buffer solution. The PL quantum yields of TEFCOONa in water and PBS buffer solution are 6.3% and 16.5%, respectively. Figure 3(b) shows the differences clearly. These features are good for avoiding cellular autofluorescence [11], when the materials were used for cellular imaging in PBS buffer solution.

Dynamic light scattering (DLS) was performed to probe the self-assemble behaviors of TEFCOONa in buffer solution. Figure 4(a) shows the results of DLS in water. The mean hydrodynamic diameter was measured to be ~85 nm, with a polydispersity of 0.287. Hydrophobic conjugated frameworks and low space charge density of the ligoelectrolyte may be the reasons of self-assemble. The morphology of the oligoelectrolyte in dry state is studied by TEM. The sample was prepared by drop-coating the polymer aqueous solution (50 μg/mL) onto Copper Grid Lacey Carbon Film, followed by evaporation in the air. As shown in Figure 4(b), the oligoelectrolyte forms nearly uniform nanospheres (~75 nm). The size measured from TEM is slightly smaller than that obtained from DLS, which should be caused by the shrinkage of the flexible chains of TEFCOONa during the drying process. In addition, these dark

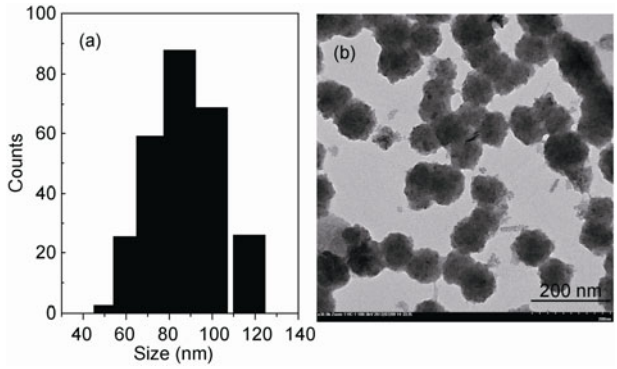

Figure 4 DLS (a) and TEM (b) images of TEFCOONa nanospheres in 10 mmol/L PBS with a scale bar of 200 nm.

cores of nanospheres correspond to the areas enriched with the conjugated segments. And the bright fluorescence from these aggregated conjugated segments can satisfy the requests for cell imaging absolutely.

3 Cell viability assays and bioimaging

Cellular viability was determined by the MTT assay which is based on the ability of the mitochondrial succinate-tetrazolium reductase system to convert 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma) to a purple-colored formazan in living cells. RAW 264.7 cells were incubated on 96 multi-well plates with 0.1 mL RPMI-1640 medium containing 10% calf serum and 1% penicillin/streptomycin and 0.5×10^4 cells per well at 37°C (5% CO_2, 95% air) for 48 h. Next, the medium without serum supplemented with indicated doses of conjugated polymers was used to incubate the cells for 24 h. Then 0.01 mL of 0.5% MTT solution was added to every well at 37°C. Four hours later, the supernatant was removed, and the product was lysed with 0.15 mL thylsulfoxide. Formazan absorbance was recorded at 490 nm using BioTek PowerWave XS2. The mean absorbance of non-exposed cells was the reference value for calculating 100% cellular viability.

3.1 Cell viability assays

Cellular morphology treated with different concentrations of TEFCOONa 0.1 μg/mL and 1 μg/mL is displayed in Figure 6(a) and (b), respectively. Figure 6(c) shows the viability of RAW 264.7 cells after being cultured with TEFCOONa in PBS solution at the concentrations of 0.1 μg/mL and 1 μg/mL for 24 h. The cell viabilities are close to 100% within the tested period of time, which indicates low cytotoxicity of materials. All of these prove the low cytotoxicity of TEFCOONa, which would be of benefit for cellular imaging *in vitro* or *in vivo* and other clinical applications.

3.2 One-photon fluorescence imaging

PANC-1 cells were incubated on 24 multi-well plates with 0.1 mL RPMI-1640 medium containing 10% calf serum and 1% penicillin/streptomycin and 0.5×10^4 cells per well at 37°C (5% CO_2, 95% air) for 24 h. Next, the DMEM medium

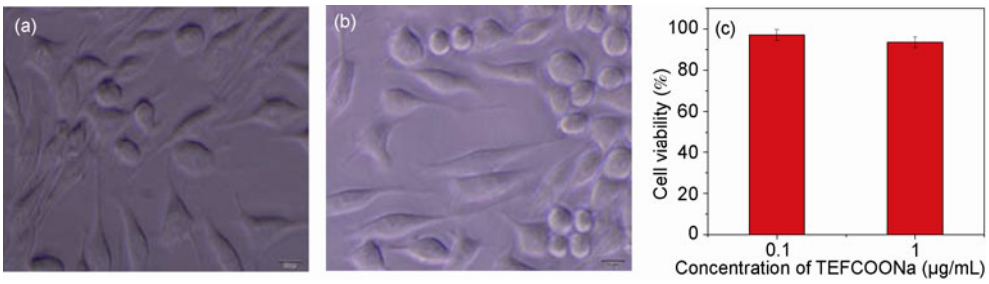

Figure 5 Characterization of living RAW 264.7 cells treated with the TEFCOONa nanospheres solution (0.1 μg/mL) (a), (1 μg/mL) (b) for 24 h and the corresponding cell viability (c) of living RAW 264.7 cells.

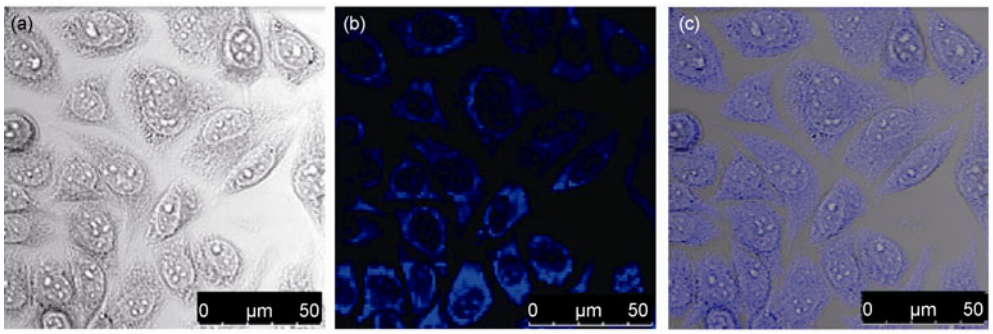

Figure 6 Fluorescence confocal microscopy images (CLSM) images of TEFCOONa (50 μg/mL) aggregates in PBS solution. (a) Bright field; (b) excited wavelength at 405 nm; (c) merging of (a) and (b).

without serum supplemented with 0.005 mg/mL conjugated polymers was used to incubate the cells at the same incubated condition. After incubation for 2 h, cells were washed three times with PBS buffer and then fixed by 75% ethanol for 20 min and further washed twice with PBS buffer solution and imaged by CLSM, Olympus, FV1000, Japan, with imaging software Fluoview FV1000.

The relatively strong PL intensity of TEFCOONa ranging from 400 to 500 nm allows collecting strong fluorescent signal. The confocal laser scanning microscopy (CLSM) images of the sample are displayed in Figure 6. Figure 6(b) is the fluorescence image and Figure 6(c) is overlapped image of Figure 6(a) and (b). It is noteworthy that the fluorescence of TEFCOONa nanospheres in cells is strong. This is ascribed to the unique structure of the oligoelectrolyte. Meanwhile, according to the CLSM images, it is noteworthy that the fluorescence from the cells treated with TEFCOONa nanospheres is strong. And the fluorescence from the nucleolus of is visible. These implicate that these nanospheres are efficiently internalized by the cells and accumulated in the cytoplasm even in the nucleolus. The results provide a facile strategy to prepare a new series of hyperbranched polymers with tagging capability and controllable properties for biological applications.

4 Conclusions

In conclusion, we take advantage of Sonogashira reaction to construct a new water-soluble oligoelectrolyte 4,4′,4″-tris (4-(9,9′-bis(3′-tert-propanoatesodium)fluorenyl-2-)ethynyl) phenylamine (TEF-COONa). The oligoelectrolyte has an effective conjugated core and anionic arms, which intrinsically forms nanospheres with an average diameter of ~75 nm according to TEM images. The inherent water miscibility and star-shaped architecture endow these nanospheres with relatively high absolute PL quantum yield in buffer solution (16.5%), allowing cell imaging in an efficient and bright fashion. As a result, this investigation provided a new candidate for biological imaging. Besides, compounds with quadrupolar (D-π-D), and three-branched architecture have the potential to be materials of Two-Photon Absorption (TPA) according to the previous reports [19–21], which is good for two-photon fluorescence imaging.

This work was supported by the National Basic Research Program of China (2009CB930600, 2012CB933301 and 2012CB723402), the Ministry of Education of China (IRT1148), the National Natural Science Foundation of China (21222404, 51173080 and 21104033), the Program for New Century Excellent Talents in University (NCET-10-0179), the Specialized Research Fund for the Doctoral Program of Higher Education (20093223110003), the Natural Science Foundation of Jiangsu Province of China (BZ2010043 and NY211003), and the Program for Postgraduates Research Innovation of Colleges and Universities of Jiangsu Province (CXLX11_0424 and CXLX11_ 0424_0414)

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