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Original article

Targeting cancer cells with biotin–dendrimer conjugates

Wenjun Yang [a], Yiyun Cheng [a,*], Tongwen Xu [b,*], Xueyuan Wang [a], Long-ping Wen [a,*]

[a] *Hefei National Laboratory for Physical Sciences at Microscale and School of Life Sciences, University of Science and Technology of China, Hefei, Anhui 230027, China*

[b] *Laboratory of Functional Membranes, Department of Chemistry, University of Science and Technology of China, Hefei, Anhui 230026, China*

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Abstract

Star-burst dendrimers represent a superior carrier platform for targeted drug delivery. Partially acetylated generation 5 (G5) polyamidoamine (PAMAM) dendrimer was conjugated with the targeting moiety (biotin) and the imaging moiety (fluoresceinisothiocyanate, FITC), and the resulting dendrimer–biotin conjugate was characterized by ^{1}H NMR, UV–vis spectrum. As revealed by flow cytometry and confocal microscopy, the bifunctional conjugate (dendrimer–biotin–FITC) exhibited much higher cellular uptake into HeLa cells than the conjugate without biotin. The uptake was energy-dependent, dose-dependent, and could be effectively blocked by dendrimer-conjugated biotin. Our results indicated that the biocompatible biotin–dendrimer conjugate might be a promising nano-platform for cancer therapy and cancer diagnosis.

Keywords: Dendrimer; Cancer targeting; Biotin; Conjugate; Nano-carrier

1. Introduction

Targeted drug delivery to cancer cells or tumor vasculature is an attractive approach to fight against cancer [1]. It can enhance the therapeutic effect while reducing or preventing toxic side effects associated with chemotherapy [2]. Current drug molecules often act poorly at these tasks, thereby requiring the use of therapeutic cocktails in clinical practice [3]. Macromolecular carriers have shown promise as an ideal targeted drug delivery system [4]. They can be employed either by the enhanced permeability and retention (EPR) strategy or as polymeric platforms with the help of a targeting moiety for selective delivery [1]. These carriers need to be biocompatible, highly soluble, stable, capable of loading large amount of anticancer drugs, and able to conjugate with the targeting molecules [5].

Dendrimers are artificial macromolecules with tree-like structures. They are hyperbranched and monodisperse three-dimensional molecules with defined molecular weights and host–guest entrapment properties [6,7]. Dendrimers are synthesized from branched monomer units in a step-wise manner, thus it is possible to precisely control their molecular properties, such as size, shape, dimension, density, polarity, flexibility, and solubility, by choosing different building/branching units and surface functional groups [8]. In general, dendrimers possess empty internal cavities and can encapsulate hydrophobic drug molecules [9]. In addition, they have a much higher surface functional group density when compared with conventional macromolecules, giving rise to their applications for enhancing the solubility of many drugs [10–16]. Furthermore, the large numbers of surface functional groups on dendrimer's outer shell can be modified or conjugated with a variety of interesting guest molecules [17,18]. These specific properties make dendrimers suitable for drug delivery systems [1,19–24]. In the recent years, increasing interest has been attracted

Abbreviations: PAMAM, poly(amidoamine); FITC, fluoresceinisothiocyanate; EPR, enhanced permeability and retention; HABA, 4-hydroxyazobenzene-2-carboxylic acid; D_2O, deuterium oxide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; FACS, fluorescence-activated cell-sorting; SMVT, sodium-dependent multi-vitamin transporter; PEG, poly(ethylene glycol); HPMA, hydroxypropylmethacrylic acid.

* Corresponding authors.
E-mail addresses: yycheng@mail.ustc.edu.cn (Y. Cheng), twxu@ustc.edu.cn (T. Xu), lpwen@ustc.edu.cn (L.-p. Wen).

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to the application of dendrimers as targeting carriers in cancer therapy [5]. It was well established that the conjugation of special targeting moieties to dendrimers can lead to preferential distribution of the cargo in the targeted tissue or cells. Examples of these special targeting moieties include sugar [25], folic acid [17,18,26–29], antibody [30,31], peptide [2] and epidermal growth factor [32,33].

Biotin, a member of the vitamin family (vitamin H), is a growth promoter of cells. Its content in cancerous tumors is significantly higher than in normal tissue. Rapid proliferations of cancer cells require extra biotin, and these cancer cells often over-express biotin-specific receptors on the cell surface. Recently, several researches showed that biotin-conjugated macromolecular carriers were able to increase the uptake of anti-cancer drugs in tumor cells [34–38]. Thus, the specific interactions between biotin and its receptors may be exploited for targeted drug delivery. In the present study, we have synthesized and characterized dendrimer–biotin conjugates and assessed their *in vitro* targeting ability to cancer cells (Scheme 1). To the best of our knowledge, the use of biotin–dendrimer conjugate as a nanodevice for cancer targeting has not been reported so far.

2. Materials and methods

2.1. Materials

G5 polyamidoamine (PAMAM) dendrimer, biotinamidohexanoic acid 3-sulfo-*N*-hydroxysulfosuccinimide ester (BAC-SulfoNHS, MW 556.8 Da) and Immunoprobe Biotinylation kit were purchased from Sigma (St. Louis, MO, USA). Acetic anhydride was a gift from School of Life Sciences, University of Science and Technology of China. Dialysis membrane (MWCO, 3500), fluoresceinisothiocyanate (FITC), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay kit, triethylamine, and dimethyl sulfoxide (DMSO) was obtained from BBI Inc. (Shanghai, China). Deuterium oxide (D_2O) was purchased from Beijing Chongxi High-Tech Incubator co., Ltd (Beijing, China). All the reagents were used as received without further purification. The cells used in this study were purchased from Keygen Co. (Nanjing, China).

2.2. Synthesis of partially acetylated PAMAM dendrimers

Partially acetylated G5 PAMAM dendrimer (Ac-G5) was synthesized according to a reported method [29]. Briefly, acetic anhydride (120% ratio of primary amine numbers of a G5 PAMAM dendrimer) was slowly added to the G5 dendrimer solution (1.39 μmol G5 dendrimer dissolved in 4 ml methanol) in the presence of triethylamine (37 μl, 1.25 equivalents of acetic anhydride). The mixtures were then stirred under N_2 atmosphere for 24 h at room temperature. Acetic acid, a byproduct of the acetylation, was removed by extensive dialysis (MWCO = 3500 Da) of the reaction mixture against PBS buffer and double-distilled water for 3 days. The obtained sample Ac-G5 was lyophilized and stored in a dry place before further modification and characterization.

2.3. Conjugation of biotin to acetylated PAMAM dendrimers

Lyophilized Ac-G5 (0.56 μmol) dissolved in 3.76 ml of double-distilled water was mixed with 12.6 mg (22.6 μM) of BAC-SulfoNHS at a molar ratio of 1:40 for 2 h. The mixture was then transferred to a dialysis membrane (MWCO = 3500 Da) and dialyzed to remove unreacted BAC-SulfoNHS. The obtained sample Ac-G5-biotin was stored in a dry place before further modification and characterization.

2.4. Measurement of biotinylation rate with 4-hydroxyazobenzene-2-carboxylic acid (HABA) assay

Briefly, the avidin/HABA reagent was prepared according to the manufacturer's instructions by adding 10 mg of avidin and 600 μl of 10 mM HABA (Sigma) to 19.4 ml PBS (pH 7.4). One hundred microliters of serially diluted Ac-G5-biotin solution was added to 900 μl of the avidin–HABA solution, and the absorbance was measured at 500 nm.

2.5. Conjugation of FITC to acetylated PAMAM dendrimers and biotinylated PAMAM dendrimers

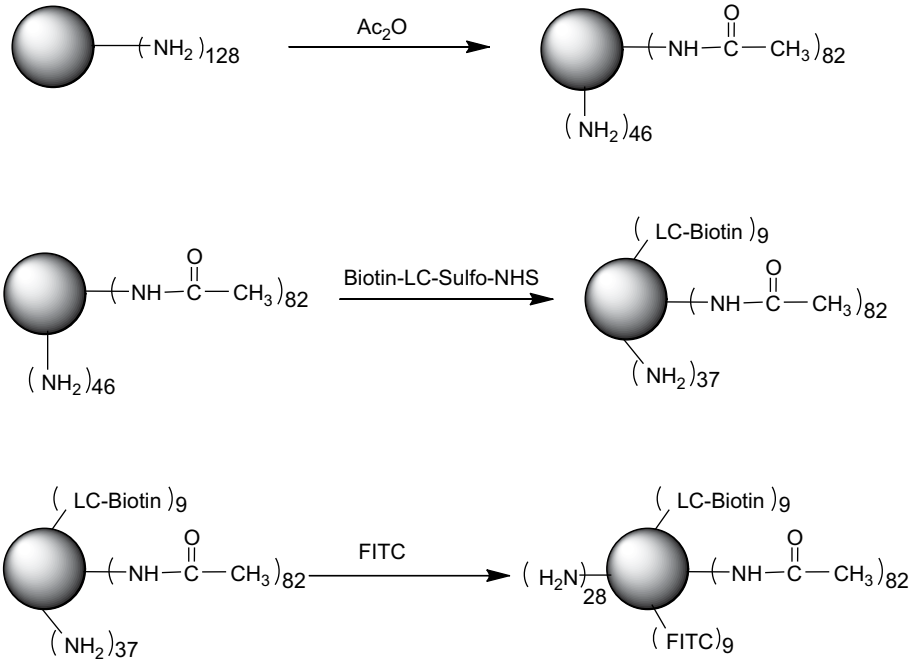
Ten molar equivalents of FITC (1.352 mg, 3.47 μmol) dissolved in DMSO (0.27 ml) was slowly added to a solution of the Ac-G5 (0.35 μmol) or Ac-G5-biotin (0.35 μmol) in distilled water (4 ml). After gentle stirring overnight at 4 °C, the reaction mixtures were purified by exhaustive dialysis against distilled water using 3500 Da MWCO membranes in a dark room for 3 days. The solutions were then lyophilized to obtain orange powders (Ac-G5-biotin–FITC and Ac-G5-FITC) for further characterization and stored in a dark place before cell level studies.

2.6. Characterization of the synthesized conjugates

^{1}H NMR spectra of the synthesized conjugates including Ac-G5, Ac-G5-biotin, Ac-G5-FITC and Ac-G5-biotin–FITC were obtained on a 500 MHz NMR spectrometer (Bruker, German) with samples dissolved in deuterium oxide (D_2O) at a concentration of 2–3 mg/ml. Acetylated rate of G5 PAMAM dendrimer can be calculated from the ^{1}H NMR spectra of these conjugates. UV–vis spectrophotometer was used to estimate the amount of FITC conjugated to each Ac-G5 or Ac-G5-biotin unit. The UV–vis spectra of Ac-G5-FITC and Ac-G5-biotin–FITC were obtained in a 0.5 ml quartz cuvette by using a Perkin–Elmer spectrophotometer.

2.7. Cell cultures and biological evaluation

Cells were grown continuously as a monolayer at 37 °C, and 5% CO_2 in Dulbecoo's modified Eagle's medium (DMEM) supplemented with streptomycin (100 μg/ml), penicillin sulphate



Scheme 1. Schematic representation of the reactions involved in the synthesis of multi-functional nanodevices based on PAMAM dendrimers for cancer cell targeting and imaging.

(100 units/ml) and 10% heat-inactivated fetal calf serum (FCS). About 4×10^4 cells/well were seeded in 24 (for FACS studies) or 96 (for confocal images) well plates 24 h before the *in vitro* targeting efficacy studies. The cells were then incubated with the conjugates (final concentrations of 0.1, 0.25, 0.5, 1, 2.5 and 5 μM for both Ac-G5-FITC and Ac-G5-biotin–FITC) and maintained at 37 or 4 °C for 2, 4, 6 and 8 h. At the end of incubation period, the medium was removed, the cells were washed three times with 0.5 ml PBS buffer, trypsinized using a standard protocol, and resuspended in 0.4 ml DMEM. For the competitive binding study, Ac-G5-biotin was added in specific concentrations (20 μM) to the medium before the conjugates were added. Specific binding properties of the conjugates were examined by flow cytometry using a fluorescence-activated cell-sorting (FACS) caliber flow cytometer (Becton Dickinson, U.S.A.), while surface and interior localization of the conjugates were determined by using a LSM510 confocal microscope (Zeiss, Germany) coupled to a Nikon inverted microscope. Cytotoxicity of the synthesized conjugate towards HeLa cells was evaluated at different concentrations (500–8000 nM) and different incubation times (24 and 48 h) by a well-established MTT assay.

3. Results and discussion

To increase the solubility and decrease the non-specific cellular uptake, the primary amine groups on the surface of PAMAM dendrimer were partially converted to acetamide moieties in the presence of acetic anhydride and triethylamine. The degree of acetylation was measured by ^{1}H NMR, using the ^{1}H proton integration method. ^{1}H NMR spectrum of the acetylated dendrimer showed the proton signal at δ 2.31 ppm, which corresponded to the methylene protons of $-CH_2C(O)-$ in G5 PAMAM dendrimer (Appendix in the supporting information). The specific signal at δ 1.88 ppm corresponded to the methyl protons of induced acetyl groups. The integration ratio of these two kinds of proton signals in the acetylated dendrimer is 1.6589, suggesting that an average of 82 acetyl groups are present on the surface of each G5 PAMAM dendrimer (Ac_{82}-G5). Ac_{82}-G5 was further biotinylated by a biotinylation reagent named BAC-SulfoNHS. The avidin/HABA assay revealed that on an average 9 biotin molecules were conjugated to each Ac_{82}-G5 molecule (Ac_{82}-G5-biotin$_9$). The acetylated and

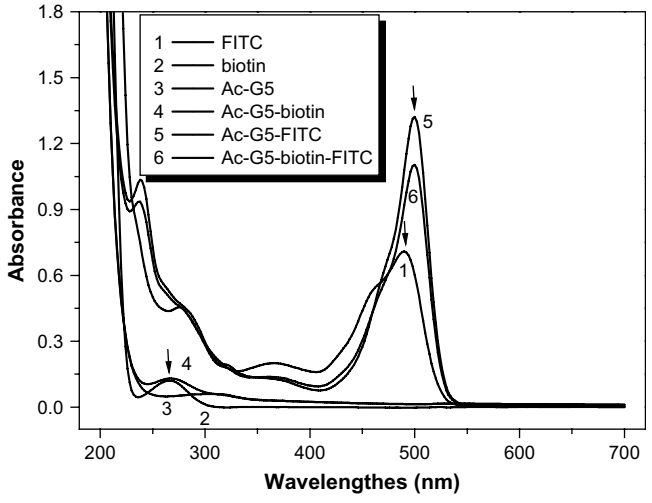


Fig. 1. UV–vis spectra of various synthesized dendrimer conjugates. The arrows at 490 and 500 nm indicate the presence of FITC in the conjugates, while the arrow at 265 nm indicates the presence of biotin molecules in the conjugates.

biotinylated dendrimer was finally labeled with the fluorescent dye FITC. To determine the number of FITC molecules successfully labeled on the surface of dendrimer, UV–vis spectrum and ^{1}H NMR analysis was performed for the products. The maximum peak of the FITC-labeled dendrimer in the visible region appeared at 500 nm (Fig. 1), shifting 10 nm from the characteristic peak of free FITC (490 nm). The same peak was also observed with FITC-labeled Ac_{82}-G5. Since Ac_{82}-G5 and Ac_{82}-G5-biotin$_9$ give no absorbance between 450 and 550 nm, the peaks obtained from the conjugates at this region would be solely from FITC molecules. The absorbance value of the conjugates at 490 nm was proportional to the amount of bounded FITC. From a calibration curve of free FITC, the approximate number of FITC molecules bound to each Ac_{82}-G5-biotin$_9$ was calculated to be 9.49. Ac_{82}-G5 and FITC (9.03 molecules) conjugate without biotin moiety was prepared to act as a control in the cancer targeting efficacy experiments. In addition, the FITC numbers conjugated to each dendrimer calculated from the integration areas of aromatic peaks at 6.44 and 7.05 ppm, which are representative of aromatic protons within the FITC molecules, were consistent with the results obtained from the UV–vis spectra. Therefore, the final products are defined as Ac_{82}-G5-biotin$_9$–FITC$_9$ and Ac_{82}-G5-FITC$_9$, respectively.

To evaluate the uptake of biotin–dendrimer conjugates to cancer cells, we conducted FACS analysis on HeLa cells treated with Ac_{82}-G5-biotin$_9$–FITC$_9$ and Ac_{82}-G5-FITC$_9$. As shown in Fig. 2a, the cells treated with Ac_{82}-G5-biotin$_9$–FITC$_9$ at a concentration of 500 nM exhibited a much higher fluorescence than that treated with Ac_{82}-G5-FITC$_9$ under the same incubation condition (4 h, 37 °C). The fluorescence from Ac_{82}-G5-FITC$_9$ treated HeLa cells was probably due to the non-specific interaction between positively charged dendrimer conjugate and the negatively charged cell membrane. Both Ac_{82}-G5-biotin$_9$–FITC$_9$ and Ac_{82}-G5-FITC$_9$ exhibited a normal dose response, with increased fluorescence from cells treated with increasing conjugate concentrations (Fig. 2b). And in all of the concentration levels tested, higher fluorescence was observed with Ac_{82}-G5-biotin$_9$–FITC$_9$ than with Ac_{82}-G5-FITC$_9$, indicating biotinylation strongly facilitates the uptake of Ac_{82}-G5-FITC$_9$ by HeLa cells.

To demonstrate the specificity of the uptake process, we performed competition experiments with Ac_{82}-G5-biotin$_9$ and Ac_{82}-G5. As shown in Fig. 2c, 20 μM of Ac_{82}-G5-biotin$_9$ effectively decreased cellular uptake of Ac_{82}-G5-biotin$_9$–FITC$_9$ to HeLa cells and reduced the cell fluorescence by more than 55%, while the same concentration of Ac_{82}-G5 had no effect

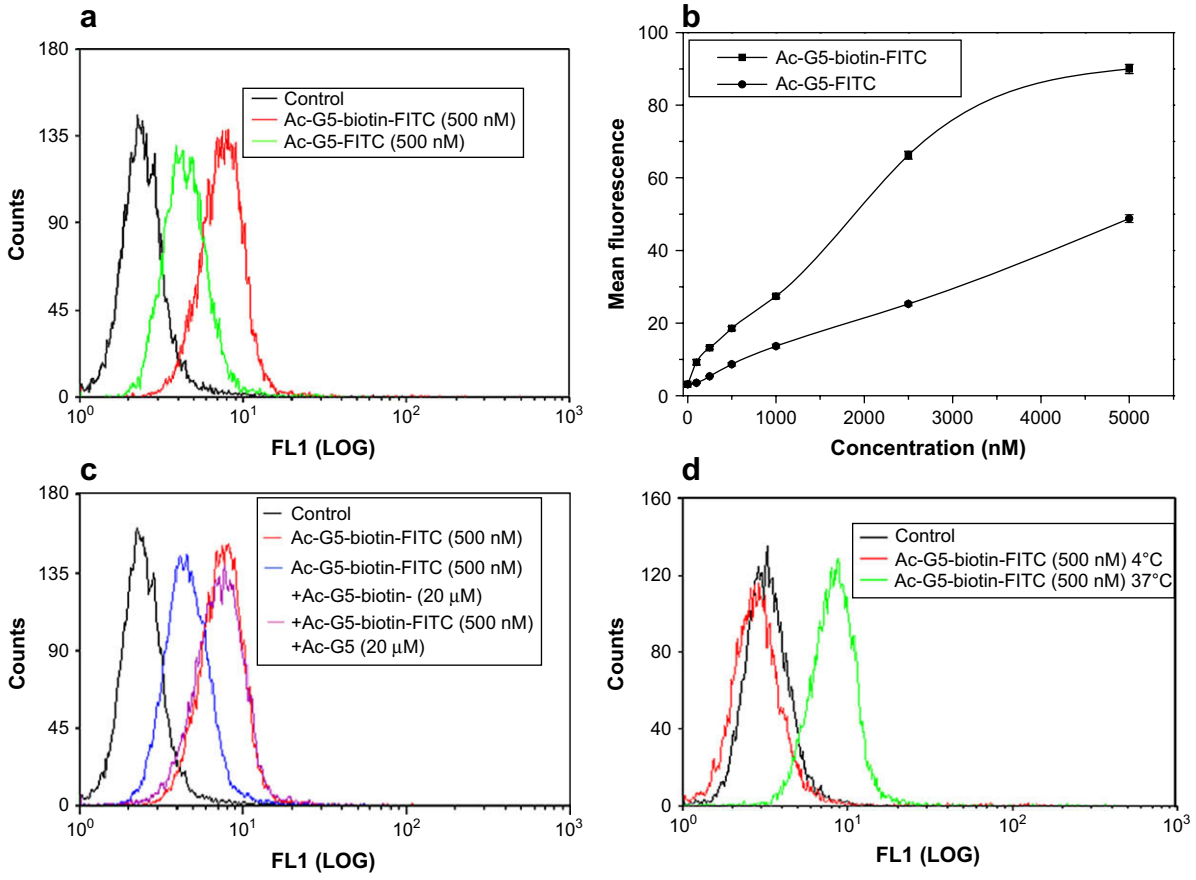


Fig. 2. *In vitro* evaluation of the uptake of the synthesized conjugates to HeLa cells determined by FACS (a), the HeLa cells were incubated with the conjugates (Ac_{82}-G5-biotin$_9$–FITC$_9$ and Ac_{82}-G5-FITC$_9$, 500 nM) for 4 h at 37 °C. (b) Dose-dependent uptake of Ac_{82}-G5-biotin$_9$–FITC$_9$ to HeLa cells, the HeLa cells were incubated with various concentrations of the conjugates for 4 h at 37 °C. (c) Competitive inhibition of the uptake of Ac_{82}-G5-biotin$_9$–FITC$_9$ to HeLa cells by Ac_{82}-G5-biotin$_9$ and Ac_{82}-G5, the HeLa cells were incubated with 500 nM Ac_{82}-G5-biotin$_9$–FITC$_9$ in the absence or presence of 20 μM Ac_{82}-G5-biotin$_9$ or Ac_{82}-G5 for 4 h at 37 °C. (d) Energy-dependent targeting of Ac_{82}-G5-biotin$_9$–FITC$_9$ to HeLa cells determined by FACS, the HeLa cells were incubated with 500 nM Ac_{82}-G5-biotin$_9$–FITC$_9$ for 4 h at 4 and 37 °C.

on cell fluorescence, indicating that the enhanced uptake of Ac_{82}-G5-biotin$_9$–FITC$_9$ conjugate to HeLa cells was mostly mediated by biotin molecules. Besides, the conjugate Ac_{82}-G5-biotin$_9$–FITC$_9$ also exhibited enhanced cellular uptake by another cancer cell lines (the KB cells, Figs. S1 in the supporting information) compared to Ac_{82}-G5-FITC$_9$, indicating that the effectiveness of this nanodevice is not limited to HeLa cell lines. We also tested the binding of the Ac_{82}-G5-biotin$_9$–FITC$_9$ and Ac_{82}-G5-FITC$_9$ to normal cell lines such as HEK 293A (primary human embryonic kidney cells transformed by sheared human adenovirus type 5 DNA) and NIH/3T3 (Mouse embryonic fibroblast cell line) cells. The binding affinities of Ac_{82}-G5-biotin$_9$–FITC$_9$ and Ac_{82}-G5-FITC$_9$ to both HEK 293A and NIH/3T3 cells are scarcely different from each other, suggesting the targeting ability of Ac_{82}-G5-biotin$_9$–FITC$_9$ to cancer cells (Fig. S2 in the supporting information).

To assess whether the cellular uptake of Ac_{82}-G5-biotin$_9$–FITC$_9$ was energy-dependent, we incubated it with HeLa cells at 4 °C and then measured cell fluorescence by FACS. As shown in Fig. 2d, no cellular uptake of Ac_{82}-G5-biotin$_9$–FITC$_9$ was detected at this temperature. This result strongly suggested that the cellular uptake of Ac_{82}-G5-biotin$_9$–FITC$_9$ requires energy and most likely occur through endocytosis.

To visualize the specific uptake of Ac_{82}-G5-biotin$_9$–FITC$_9$ by HeLa cells, we performed confocal microscopy studies on HeLa cells treated with the conjugates. Consistent with the FACS analysis results, Ac_{82}-G5-biotin$_9$–FITC$_9$ treated cells was much more fluorescent than Ac_{82}-G5-FITC$_9$ treated cells (Fig. 3a–c), indicating a higher level of uptake. The distribution of fluorescence inside the cells appeared to be non-uniform, with higher fluorescence in and around the nucleus (Fig. 3d). These results agreed well with the published reports

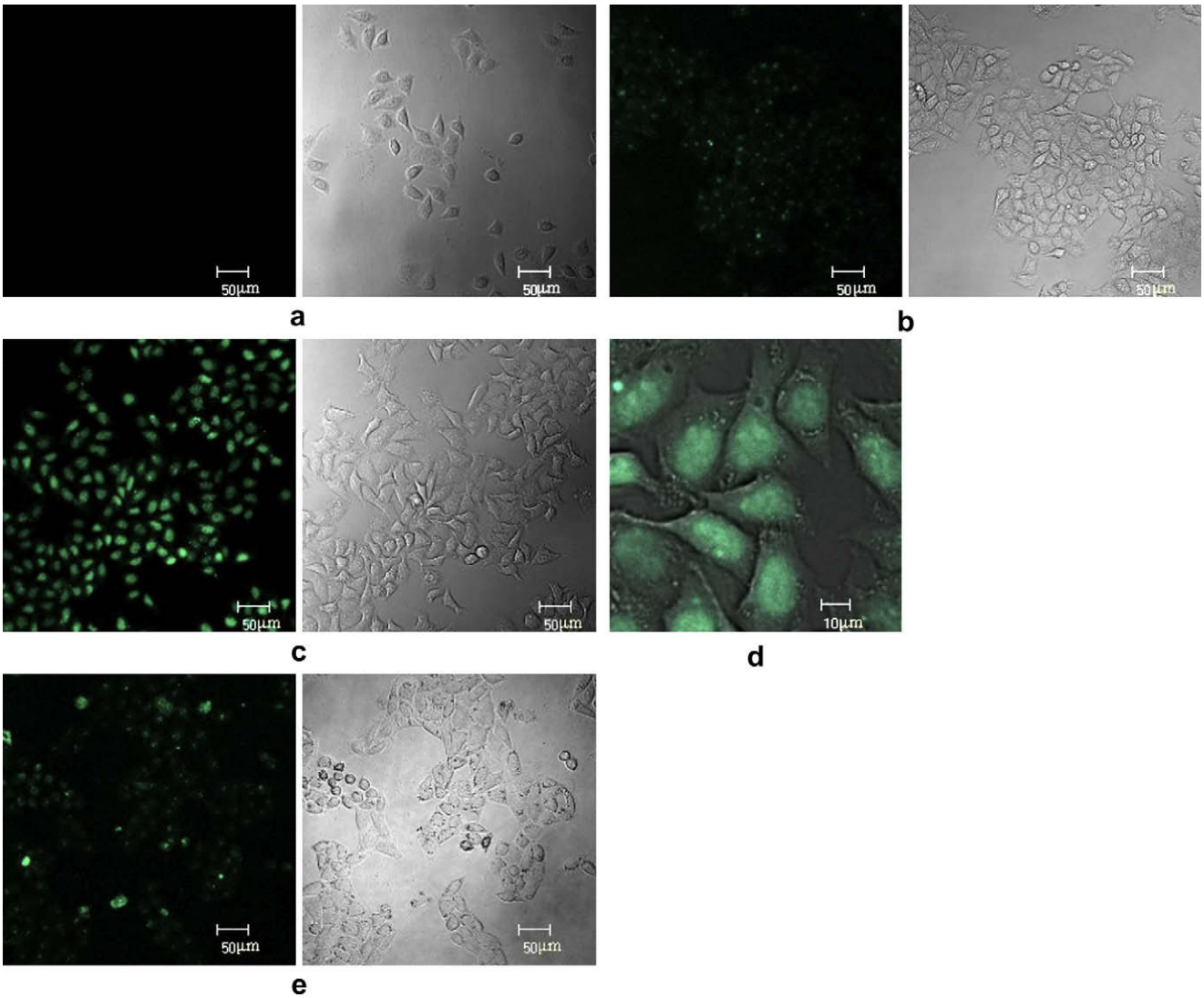


Fig. 3. *In vitro* evaluation of targeting the synthesized conjugates to HeLa cells determined by confocal microscopy. The HeLa cells were incubated with PBS (a), Ac_{82}-G5-FITC$_9$, 500 nM (b) and Ac_{82}-G5-biotin$_9$–FITC$_9$, 500 nM (c) for 24 h at 37 °C. (d) Local amplification of (c). A significant difference of fluorescence from the Ac_{82}-G5-biotin$_9$–FITC$_9$, Ac_{82}-G5-FITC$_9$, and PBS control is observed. The conjugate Ac_{82}-G5-biotin$_9$–FITC$_9$ localizes both in the periphery of the cell membrane and at the cytoplasm of the cell. (e) Competitive inhibition of targeting Ac_{82}-G5-biotin$_9$–FITC$_9$ to HeLa cells by Ac_{82}-G5-biotin$_9$ determined by confocal microscopy. The HeLa cells were incubated with 500 nM Ac_{82}-G5-biotin$_9$–FITC$_9$ in the presence of 20 μM Ac_{82}-G5-biotin$_9$ for 24 h at 37 °C.

indicating that dendrimers were efficient transfection reagents for DNA [39]. As would be expected, the addition of Ac_{82}-G5-$biotin_9$ to the incubation medium significantly reduced the observed fluorescence in HeLa cells treated with Ac_{82}-G5-$biotin_9$–$FITC_9$ (Fig. 3e).

To confirm the biocompatibility of the biotin–dendrimer conjugate to HeLa cells, the product Ac_{82}-G5-$biotin_9$–$FITC_9$ was examined on HeLa cells by a MTT assay. As shown in Fig. 4, Ac_{82}-G5-$biotin_9$–$FITC_9$ displayed high cell viability (more than 80%) in the concentration range from 500 to 8000 nM after 24 h (Fig. 4) or 48 h (data not shown) of incubation. Generally, cytotoxicity of amine-terminated PAMAM dendrimers is known to arise from their cationic charged surface. These results indicate that the acetylation in the first step is successful and the addition of biotin and FITC molecules in the following steps does not bring any extra cytotoxicity. Overall, the cytotoxicity of Ac_{82}-G5-$biotin_9$–$FITC_9$ is minimal and the created multi-functional conjugate is biocompatible as a novel platform for cancer therapy and diagnosis.

Recently, more attention has been paid to the sodium-dependent multi-vitamin transporter (SMVT) which is responsible for the transfer of vitamins including biotin into many cancer cell lines [40–42]. Previous studies have shown that poly(ethylene glycol) (PEG) and biotin conjugates were able to target SMVT on the cell membrane of human carcinoma cells and substantially improve the delivery and anti-cancer activity of camptothecin, a well-established anti-cancer drug [43]. Similarly, biotinylated hydroxypropylmethacrylic acid (HPMA) polymers were successfully targeted to ID8 cells, the murine cancer cell lines, through the interactions between biotin and biotin receptors [34]. Our results in the present study agreed well with previous results and demonstrated that biotin molecules which were conjugated to the surface of dendrimers significantly enhanced the uptake level of dendrimer–FITC conjugates into cancer cells, probably by transporter-mediated specific cellular uptake. Of course, the attractive properties of dendrimers, such as extremely low polydispersity, regular and high degree of branching, multi-valency, nano-sized scale, globular architecture and well-defined molecular weight, together with the excellent targeting efficiency of biotin–dendrimer conjugates, endue these nanodevices with unique advantages over traditional macromolecules such as PEG–biotin and HPMA–biotin conjugates.

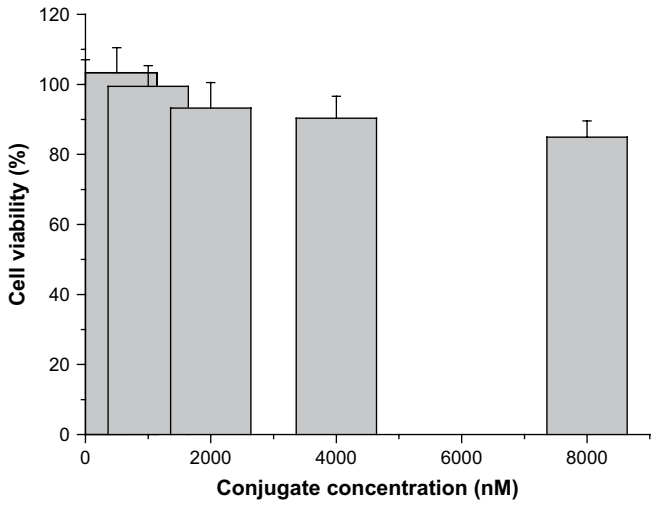


Fig. 4. Cytotoxicity of Ac_{82}-G5-$biotin_9$–$FITC_9$ towards HeLa cells after 24 h of incubation determined by an MTT assay.

4. Conclusions

In summary, partially acetylated PAMAM dendrimer with targeting and imaging moieties were successfully synthesized and characterized. The multi-functional conjugate Ac_{82}-G5-$biotin_9$–$FITC_9$ exhibited much higher cellular uptake into HeLa cells than the conjugate without biotin molecules. The uptake process was energy-dependent, showed an expected dose–response curve, and could be effectively blocked by biotin–polymer conjugates. Our results indicated that biotin–dendrimer conjugate might be a promising nano-platform for cancer targeting and cancer diagnosis. Current work of using biotin–dendrimer conjugates as scaffolds for preparing cancer therapeutics is limited to "proof-of-concept" studies, and a long road lies ahead to actually use these nanodevices in clinical practice.

Acknowledgements

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Appendix. Supplementary material

Supplementary data associated with this article can be found, in the online version at doi:10.1016/j.ejmech.2008.04.021.

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