pH-responsive endosomolytic pseudo-peptides for drug delivery to multicellular spheroids tumour models

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Abstract

Endosomolytic polymers can aid in the endosomal release of therapeutics to improve intracellular drug delivery. pH-responsive biomimetic pseudo-peptides were synthesised by grafting L-phenylalanine onto the pendant carboxylic acids of a polyamide, poly(L-lysine isophthalamide). PP-75 (stoichiometric L-phenylalanine grafting of 75 mol%) was determined to have the best endosomolytic property. The mean hydrodynamic size of PP-75 decreased with lower pH as the polymers adopted a more compact conformation due to protonation of acidic groups and increase in hydrophobicity. PP-75 was demonstrated to deliver model drugs effectively in three dimensional (3D) magnetic HeLa multicellular spheroids used as *in vitro* tumour models. These spheroids can be isolated easily and quickly by magnetic separation. Due to its relatively small size, PP-75 was able to penetrate from the exterior to the interior of these spheroids and was internalised by the cells in the spheroids. It could retain its pH-mediated membrane-lytic capability in 3D drug delivery by releasing internalised calcein from intracellular endosomes in the tumour models. Furthermore, cell viability results suggest that PP-75 showed no significant cytotoxicity towards cells in the spheroids. The pH-responsive PP-75 can potentially enhance the extracellular and intracellular delivery of therapeutics in tumours.

Keywords: drug delivery; pH-responsive polymer; L-phenylalanine; pseudo-peptide; multicellular spheroids; magnetic separation

1. Introduction

One of the main aims of drug delivery research is the efficient intracellular delivery of therapeutics, particularly macrodrugs such as proteins and nucleic acids. Mammalian cells usually internalise macromolecular prodrugs through endoctyosis which results in endosomal localisation. After cellular uptake, internalised drugs face several obstacles such as lysosomal degradation before they can reach their target organelles or cell nuclei [1]. To prevent lysosomal degradation, drugs entrapped in endosomes must be able to 'escape' into the cytosol before the endosomes fuse with lysosomes. One strategy for endosomal release is through membrane disruption using pH-responsive polymers [2]. These polymers can undergo pH-mediated coil-globule changes in conformation and this property enhances their membrane disruptive behaviour [3-7].

A class of biodegradable, pH-responsive polymers has been recently developed to mimic factors that enable efficient viral transfection. The parent polymer is a polyamide, poly(L-lysine isophthalamide), and hydrophobic amino acids were grafted onto its pendant carboxylic acid groups to manipulate its amphiphilicity and structure [8, 9]. Recent studies indicated that L-phenylalanine grafted polymers have vastly superior membrane-disruptive activity at endosomal pHs and could be used for intracellular drug delivery [10, 11].

Another important aspect of drug delivery is the transport of drugs through extracellular barriers before they reach the cell surface. Penetration of chemotherapeutics is often a problem for efficacious cancer therapy [12]. Ideally, the drug should reach all cells in a tumour after leaving the vasculature. However, high interstitial pressure within tumours [13], diffusion limitations and the extracellular matrix [14] present significant obstacles to effective drug delivery. Therefore

relatively high drug concentrations were frequently used to overcome these problems. This inevitably led to toxic side effects in patients [15].

Drug delivery systems serve to reduce the systemic toxicity by enhancing the delivery of therapeutics to specific diseased sites at a lower dose. However these systems are often studied using two dimensional (2D) cell monolayers which cannot reproduce the complex three dimensional (3D) environment in tissues or organs. To better model the actual *in vivo* conditions, 3D multicellular spheroids were developed and they have been used as avascular tumour models for evaluating small molecule [16] or nanoparticle [17, 18] delivery. Magnetic multicellular spheroids could be generated from magnetically labelled cells. These spheroids can be easily separated using magnetic separators and could be useful for various applications such as drug screening and toxicity assays [19-21].

In this study, the relationship between pH and hydrodynamic size of L-phenylalanine grafted polymers was examined. Furthermore, the potential of using these grafted polymers for drug delivery in 3D magnetic tumour spheroids was qualitatively and quantitatively assessed using confocal microscopy and flow cytometry respectively. The non-specific cytotoxicity of the polymers towards cells in the multicellular spheroids was evaluated as well.

2. Materials and methods

2.1. Materials

2.1.1. Materials for polymer synthesis

Isophthaloyl chloride, fluorescein 5-isothiocyanate (FITC), *N,N'*-dicyclohexylcarbodiimide (DCC), 4-dimethylaminopyridine (DMAP), *N,N'*-dimethylformamide (DMF), potassium carbonate and triethylamine were purchased

from Sigma-Aldrich (Dorset, UK). L-lysine methyl ester dihydrochloride and dimethyl sulfoxide (DMSO) were obtained from Fisher (Loughborough, UK). L-phenylalanine methyl ester hydrochloride was purchased from Alfa Aesar (Heysham, UK).

2.1.2. Materials for cell culture and drug delivery studies

Accutase®, biotinamidohexanoic acid N-hydroxysuccinimide ester (BiotinSE), calcein, Dulbecco's modified Eagle medium (DMEM), Dulbecco's phosphate buffered saline (D-PBS), penicillin-streptomycin solution (10,000 U mL^{-1} penicillin, 10 mg mL^{-1} streptomycin) and trypsin–EDTA solution (0.5 g L^{-1} porcine trypsin and 0.2 g L^{-1} EDTA·4Na) were purchased from Sigma-Aldrich (Dorset, UK). Streptavidin MagneSpheres paramagnetic particles (1 mg mL^{-1}) were purchased from Promega (Southampton, UK). Foetal bovine serum (FBS) and propidium iodide (Invitrogen P3566, 1 mg mL^{-1}) were purchased from Invitrogen (Paisley, UK).

2.2. Polymer synthesis and characterisation

PP-75 was synthesised by grafting L-phenylalanine onto poly(L-lysine isophthalamide) according to the previously reported procedure [8, 10, 22-24] using DCC as a coupling agent and DMAP as a hyperacylation catalyst [25, 26]. Here, the number denotes the stoichiometric molar percentage of L-phenylalanine relative to the pendant carboxylic acid groups of poly(L-lysine isophthalamide) ($[NH_2]/[COOH]$). FTIR spectra of poly(L-lysine isophthalamide) and PP-75 were recorded on a Nicolet Nexus FTIR spectrometer (Thermo Fisher Scientific, USA). FTIR (acid form): 3294 cm^{-1} (N–H str and O–H str), 1720 cm^{-1} (C=O acid str), 1634 cm^{-1} (amide band I), 1528 cm^{-1} (amide band II), 1274, 1088 cm^{-1} (C–O str). The molecular weight and polydispersity of poly(L-lysine isophthalamide) (M_w = 35,700, polydispersity = 1.99)

were determined using an aqueous gel permeation chromatography (GPC) system (Viscotek, UK), which was calibrated with polyethylene glycol standards. ^{1}H-NMR spectra of poly(L-lysine isophthalamide) and its derivative polymer PP-75 were obtained in d_6-DMSO on a Bruker Advance 500 MHz NMR spectrometer (Bruker Biospin GmbH, Germany). The degree of L-phenylalanine grafting of PP-75 (63.2 mol%) was measured from its ^{1}H-NMR spectrum, and used to calculate its molecular weight (M_n = 24.9 kDa).

FITC-NH-$(CH_2)_2$-NH_2 was prepared from reaction of FITC with ethylene diamine using dibutyltin dilaurate as a catalyst. The fluorescent polymer PP-75-FITC (1 mol% FITC) was prepared by coupling FITC-NH-$(CH_2)_2$-NH_2 to the carboxylic acid groups of PP-75 using standard DCC/DMAP mediated coupling techniques [10].

2.3. Dynamic light scattering

The hydrodynamic diameters of PP-75 were investigated using a PDDLS/Batch dynamic light scattering (DLS) platform equipped with a PD2000DLS detector (Precision Detectors, USA). The polymer aqueous solutions at 5 mg mL^{-1} were prepared in 0.1 M buffers at specific pHs and allowed to equilibrate for 48 h. The samples were filtered through 0.45 μm pore size filters, and then the measurements were conducted in a 1.0 mL quartz cuvette using a diode laser of 800 nm at a scattering angle of 90°.

2.4. Spheroid culture

HeLa human cervix adenocarcinoma cells were cultured in DMEM supplemented with 10% FBS, 100 U mL^{-1} penicillin and 100 μg mL^{-1} streptomycin and maintained in a 5% CO_2 humidified atmosphere at 37°C. Magnetically labelled

HeLa cells were prepared using a previously developed method [27]. Briefly, HeLa cells were treated with 750 μM BiotinSE in D-PBS at room temperature for 30 min. 0.05 mg mL^{-1} streptavidin paramagnetic particles were added to biotinylated HeLa cells and vortexed for 15 s to ensure mixing of particles and cells. To generate magnetic spheroids [19], 15 μL drops containing 500 magnetically labelled HeLa cells each were added onto a culture dish cover lid. The lids were then inverted and placed on top of the dish. 10 mL of PBS was added to prevent evaporation of the hanging drops which were cultured in a 5% CO_2 humidified atmosphere at 37°C. Six day old spheroids were harvested for further studies as described below.

2.5. 3D drug delivery to spheroids

For the concentration-dependent studies using PP-75-FITC, HeLa spheroids were treated with different PP-75-FITC concentrations in serum-free DMEM for 1 h at 37°C. For the time-dependent studies, HeLa spheroids were treated with 1 mg mL^{-1} PP-75-FITC for different durations. PP-75-FITC was then removed and the spheroids were washed with D-PBS and complete DMEM was added. The spheroids were then further incubated at 37°C for 3.5 h before they were analysed using either confocal microscopy or flow cytometry as depicted below.

For the study using calcein as a model drug, HeLa spheroids were treated with different calcein concentrations with or without PP-75 (1 mg mL^{-1}) in serum-free DMEM for 1 h at 37°C. Thereafter the spheroids were further incubated for 3.5 h at 37°C in complete DMEM before they were analysed using either confocal microscopy or flow cytometry.

2.6. Confocal laser scanning microscopy

After the spheroids were treated according to the protocol described above, they were placed on 35 mm glass-bottom culture dishes (MatTek) and imaged using a Leica SP5 confocal laser scanning microscope. Both the PP-75-FITC and calcein were excited using the 488 nm laser line. Optical sections (1 μm thick) were imaged until the fluorescence was lost. These images were then compiled into a 3D projection.

2.7. Flow cytometry

40–50 six day old HeLa spheroids were used for each experimental condition as described above. Thereafter HeLa spheroids were separated using a Dynal MPC-S magnetic particle concentrator (Invitrogen). After D-PBS wash, the spheroids were enzymatically dissociated with Accutase®. The dissociated individual cells were further washed with D-PBS and resuspended in D-PBS with 2.5 μg mL^{-1} propidium iodide. The cells were then analysed by flow cytometry (BD FACSCan). 10,000 events were recorded and the data was analysed using CellQuest. FITC fluorescence intensities of 20 arbitrary units (a.u.) and above were regarded as FITC-positive (+), as the control sample recorded fluorescence intensities of less than 20 a.u. Cell viability was assessed by gating cell populations showing negligible propidium iodide fluorescence.

3. Results and discussion

The hydrodynamic size of PP-75 was measured using DLS and it showed a pH dependent size change. At pH 6.0, PP-75 has a mean hydrodynamic diameter of 17 nm (Fig. 1a). The hydrodynamic size increases as the pH increases with a mean diameter of 37 nm at the physiological pH of 7.4, as shown in Fig. 1b. This pH

dependent behaviour occurred as PP-75 alters its conformation according to pH changes. The hydrophobicity of the polymer increases as the pH decreases with the protonation of free carboxylic acid groups and growing hydrophobic interactions between the grafted L-phenylalanine, resulting in an even more compact structure [8].

PP-75, the L-phenylalanine grafted polymer with the most promising property for endosomal membrane disruption [10], was selected for 3D delivery studies in magnetic HeLa tumour spheroids. PP-75 labelled with fluorescein 5-isothiocyanate (PP-75-FITC) was incubated with magnetic HeLa multicellular spheroids and confocal microscopy was used to study the distribution of the fluorescent polymer in the spheroids. Fig. 2 shows that the 3D delivery of PP-75-FITC is dependent on both polymer concentration and incubation duration. More polymers can be delivered into the spheroids when there is a higher polymer concentration or longer incubation time, as shown by the stronger fluorescence. The images in the Y-Z axis show that the polymers were able to penetrate into the spheroids.

The use of confocal microscopy to visualise polymer penetration into the spheroids suffers from an imaging depth limitation due to the loss of fluorescent signal and increased background at depths greater than 100–200 μm [28]. To overcome this constraint and to obtain a more quantitative analysis, flow cytometry was used for further studies. HeLa spheroids which have been treated with PP-75-FITC were enzymatically dissociated into individual cells and the cells were then analysed using a flow cytometer.

Quantitative data in Fig. 3 further proved that the 3D delivery of PP-75, shown as the proportion of FITC positive cells and mean FITC fluorescence intensity of the cell population, is dependent on both the polymer concentration and incubation duration with the polymer. Compared to HeLa cells grown as a monolayer, higher

polymer concentrations were needed to achieve a significant distribution in the HeLa spheroids. When HeLa cell monolayers were treated with 0.05 mg mL^{-1} of PP-75-FITC for 1 h, 74% of cells had taken up the fluorescent polymer. With increasing polymer concentration to 0.1 mg mL^{-1}, almost all monolayer cells (97%) were FITC-positive. As comparison, treatment with 0.05 mg mL^{-1} of PP-75-FITC for 1 h only achieved 19% cellular uptake of the polymer in HeLa spheroids. The proportion of FITC-positive cells was saturated at 93% with increasing polymer concentration up to 5 mg mL^{-1} (Fig. 3a). When the incubation duration with PP-75-FITC (1 mg mL^{-1}) was extended from 15 min to 2 h, the proportion of FITC-positive cells in the spheroids increased gradually from 34% to 83% (Fig. 3 c). There is less difficulty with diffusion for cell monolayers, while the delivery of polymers into the spheroids faced diffusion limitations and additional barriers such as the extracellular matrix [14]. Therefore higher polymer concentrations are required for efficient delivery in spheroids. This is likely the same situation for effective polymer delivery *in vivo*.

Cell viability in the multicellular spheroids was analysed by flow cytometry using the propidium iodide exclusion assay to evaluate the non-specific cytotoxicity of PP-75-FITC towards the cells in the spheroids. Propidium iodide can enter non-viable cells with permeable membranes and binds with intracellular nucleic acids by intercalation. As shown in Fig. 4a, the polymer was not drastically toxic to HeLa cells at concentration up to 4 mg mL^{-1} after 1 h of treatment. Fig. 4b shows that PP-75-FITC (1 mg mL^{-1}) only had a relatively low level of cytotoxic effect with extended incubation time to 2 h. These results suggest that PP-75 will probably have a similar toxicity profile, since the degree of FITC labelling (1 mol%) is kept low to avoid significant modulation of the polymer properties and fluorescence quenching [10].

To study whether PP-75 still retains its membrane-lytic activity in 3D delivery, magnetic HeLa spheroids were treated with free calcein (as a model drug) and PP-75. Calcein, which has been internalised by cells, is trapped within endosomes and undergo self-quenching with reduced fluorescence when excited with lasers. This quenching effect could be reduced when calcein is released from the endosomes and is freely dispersed within the cytoplasm [29].

The confocal images (Fig. 5c, d) showed that more calcein was released from endosomes when PP-75 was co-incubated with it. Flow cytometric analysis further confirmed that PP-75 was able to help attain higher intracellular calcein fluorescence within the spheroids (Fig. 6). What is even more significant is that PP-75 (with 2 mg mL^{-1} calcein) was able to achieve higher calcein fluorescence compared to the 4 mg mL^{-1} free calcein control. This could mean that lower drug concentrations can be used in polymer-mediated delivery. Non-specific cytotoxicity and side effects of drugs could then be minimised.

In the study above, PP-75 was able to penetrate efficiently into the 3D multicellular spheroids and achieve a reasonable concentration in the spheroids (Fig. 2 and 3). When the polymers were internalised, they were able to disrupt endosomal membranes for effective intracellular release of endocytosed calcein (Fig. 5 and 6). The transport of PP-75 in the spheroids might be less obstructed due to its small size (Fig. 1), which makes it easier to diffuse through the extracellular space. Studies have shown that penetration of nanoparticles into the core of multicellular spheroids was limited to particles smaller than 100 nm [17, 18]. It was demonstrated that adeno-associated viral vectors (~25 nm in diameter) can penetrate human solid tumour tissue *in vivo* more efficiently than adenoviral vectors (~100 nm in diameter) [30], which could be attributed at least in part to these differences in particle size. In addition,

grafting with the hydrophobic amino acid L-phenylalanine can facilitate effective cell surface binding, which is necessary for uptake of the polymer.

Fig. 1 shows that PP-75 formed a compact structure (37 nm in diameter at pH 7.4) stabilised by hydrophobic association. The mean hydrodynamic diameters of these polymeric nanoparticles gradually reduced to 17 nm with pH decreasing to 6.0. It is known that *in vivo* tumours have lower extracellular pH than normal tissues [31]. Moreover, pH gradients exist within spheroids and tumours with the pH decreasing from the exterior to the interior [32]. As PP-75 migrated through the spheroids, it experienced decreasing pH and could undergo further size reduction. This could have actually aided in the penetration of PP-75 towards the core of the multicellular spheroids and resulted in a higher proportion of cells which internalised the polymer.

This work represents the first time that magnetic multicellular spheroids were used as tumour models in drug delivery research. These spheroids can be easily separated using a magnetic separator within a few seconds without the need for centrifugation. This allows for facile separation of spheroids after incubation with drugs or drug delivery systems for further examination using other analytical techniques such as confocal microscopy and flow cytometry used in this study. As mentioned previously, the magnetic property of these spheroids will make them suitable for large scale or high throughput studies requiring large amounts of spheroids and quick separation for subsequent investigations [19]. Furthermore, recent studies also showed that 3D multicellular spheroids have great potential for predicting *in vivo* experimental results in drug delivery [33].

4. Conclusions

In this current study, it was shown that the pH-responsive, biodegradable and biomimetic polymers grafted with L-phenylalanine have significant potential to be used to deliver therapeutics. In particular, the endosomolytic polymer, PP-75 (63.2 mol% L-phenylalanine, M_n = 24.9 kDa), shows a pH-dependent change in its hydrodynamic size. The effective penetration of PP-75 into tumour models and its ability to release internalised model drugs within cells in tumour spheroids suggest that it could be studied in *in vivo* or clinical research to further evaluate and realise its potential for delivering therapeutics to tumours.

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Figure Captions

Figure 1. Mean hydrodynamic diameters of PP-75 at 5 mg mL^{-1} in 0.1 M buffers. (a) Size distribution at pH 6.0. (b) pH-dependent mean hydrodynamic diameters. Error bars represent standard deviations of four samples.

Figure 2. Confocal microscopy images of HeLa spheroids treated with PP-75-FITC at (a, b) 0.05 mg mL^{-1} for 1 h, (c, d) 4 mg mL^{-1} for 1 h, (e, f) 1 mg mL^{-1} for 2 min and (g, h) 1 mg mL^{-1} for 2 h. Samples were imaged after 3.5 h further incubation. (a, c, e, g) show the image in the X-Y axis, while (b, d, f, h) show the image in the Y-Z axis.

Figure 3. Flow cytometric analysis of cellular uptake of PP-75-FITC in HeLa multicellular spheroids. (a, b) Concentration-dependent uptake of PP-75-FITC after treatment for 1 h. (c, d) Time-dependent uptake of PP-75-FITC at 1 mg mL^{-1}. Samples were tested after 3.5 h further incubation. (a, c) show the proportion of FITC-positive cells, while (b, d) show the relative mean fluorescence intensity (MFI) of cell population expressed in arbitrary units (a.u.). Relative MFI = ($MFI_{sample}/MFI_{control}$). 40-50 spheroids were used for each experimental condition and the spheroids were enzymatically dissociated for subsequent flow cytometric analysis.

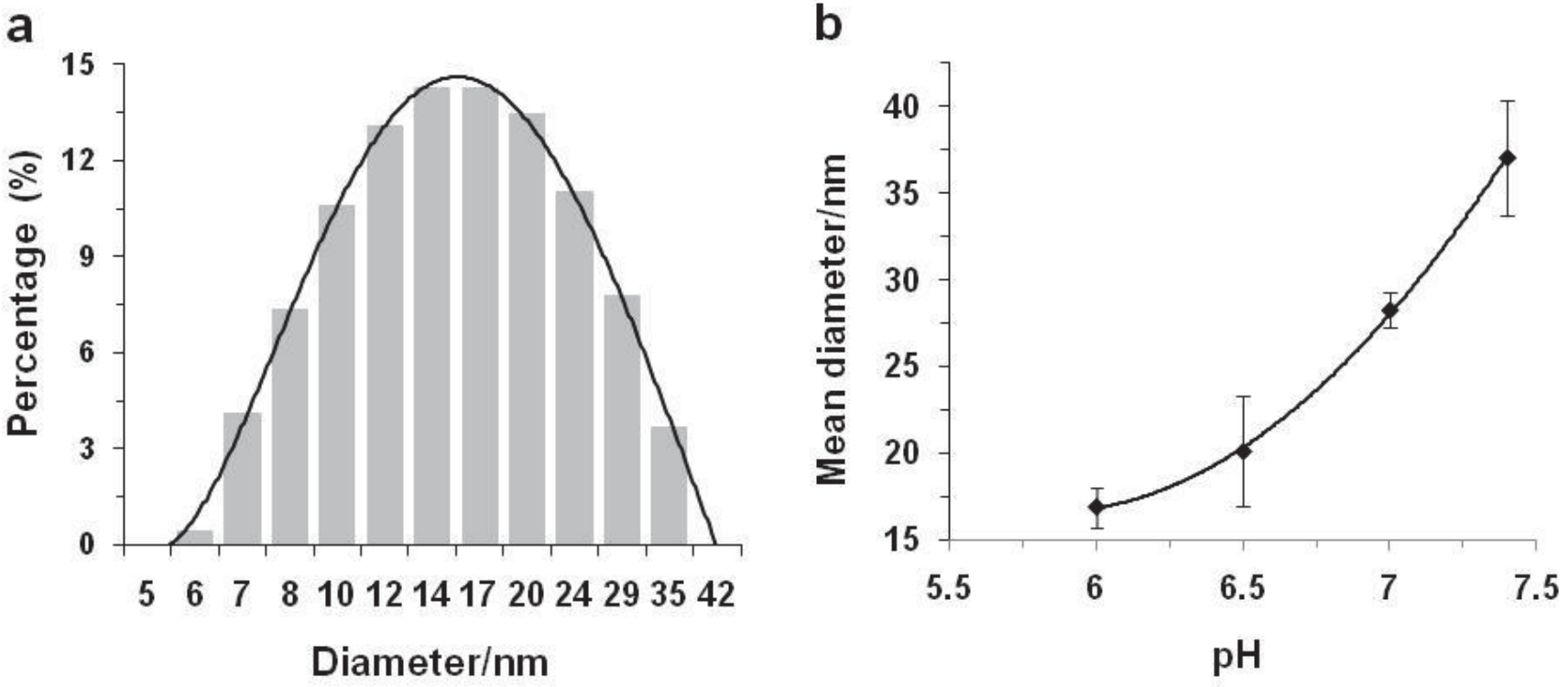
Figure 4. Concentration- and time-dependent cell viabilities of HeLa spheroids treated with PP-75-FITC. (a) PP-75-FITC concentration-dependent studies. Spheroids were treated with various concentrations of PP-75-FITC for 1 h with 3.5 h further incubation in fresh complete culture media. (b) PP-75-FITC treatment duration-dependent studies. Spheroids were treated with 1 mg mL^{-1} PP-75-FITC for various durations with 3.5 h further incubation in fresh complete culture media. Relative cell viabilities were expressed in percentage based on the control without polymer treatment. 40-50 spheroids were used for each experimental condition and the spheroids were enzymatically dissociated for subsequent flow cytometric analysis using the propidium iodide exclusion assay.

Figure 5. Confocal microscopy images of HeLa spheroids treated with (a, b) 2 mg mL^{-1} calcein for 1 h, and (c, d) 2 mg mL^{-1} calcein with 1 mg mL^{-1} PP-75 for 1 h. Samples were imaged after 3.5 h further incubation. (a, c) show the image in the X-Y axis, while (b, d) show the image in the Y-Z axis.

Figure 6. Flow cytometric analysis of cellular uptake of calcein and PP-75 in HeLa spheroids. Spheroids were treated with various concentrations of calcein with or without PP-75. Samples were tested after 3.5

h further incubation. Relative mean fluorescence intensity (MFI) of cell population was expressed in arbitrary units (a.u.). Relative MFI = ($MFI_{sample}/MFI_{control}$). 40-50 spheroids were used for each experimental condition and the spheroids were enzymatically dissociated for subsequent flow cytometric analysis.

Figure 1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Percentage (%)
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Diameter/nm
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Figure 2

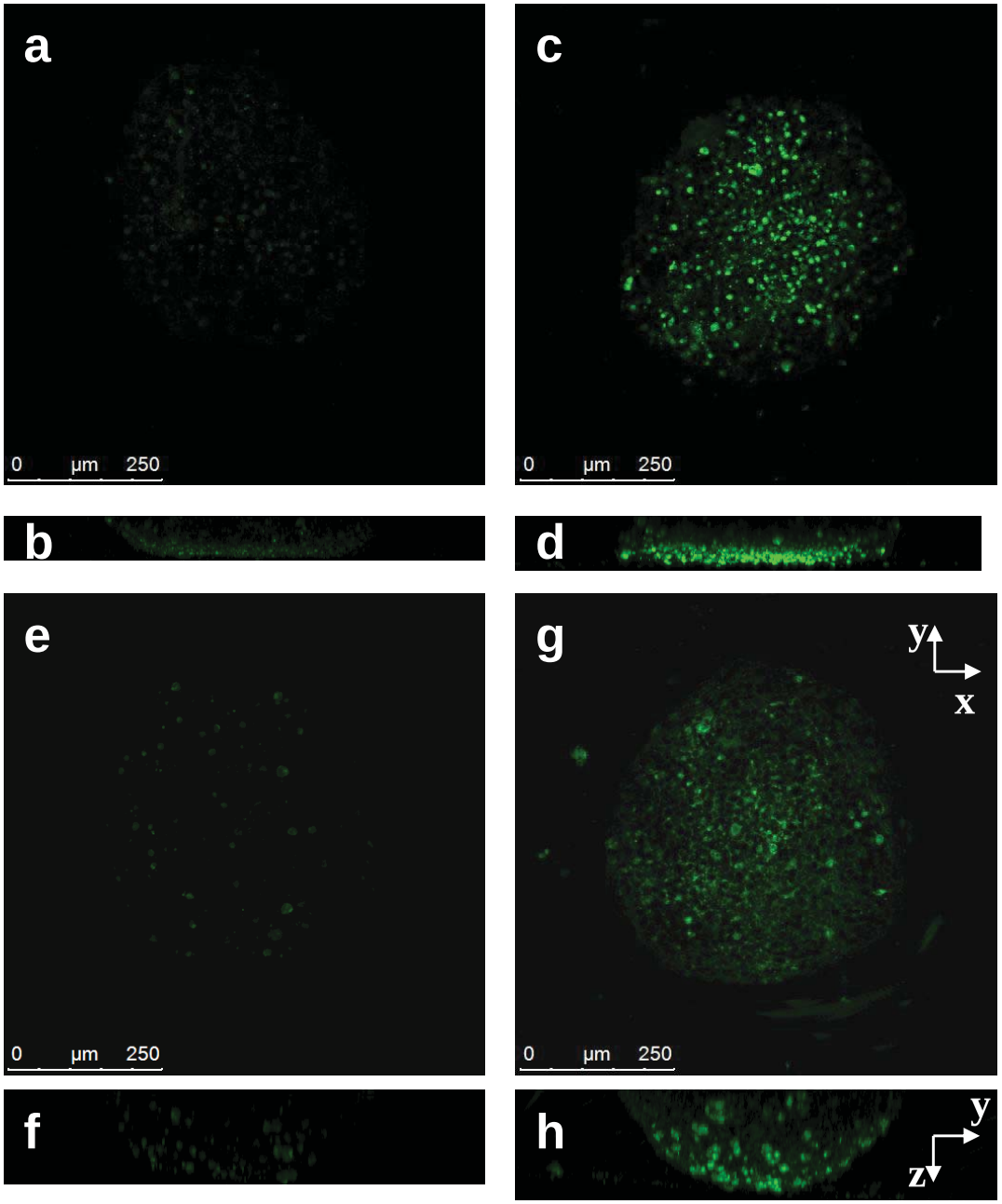
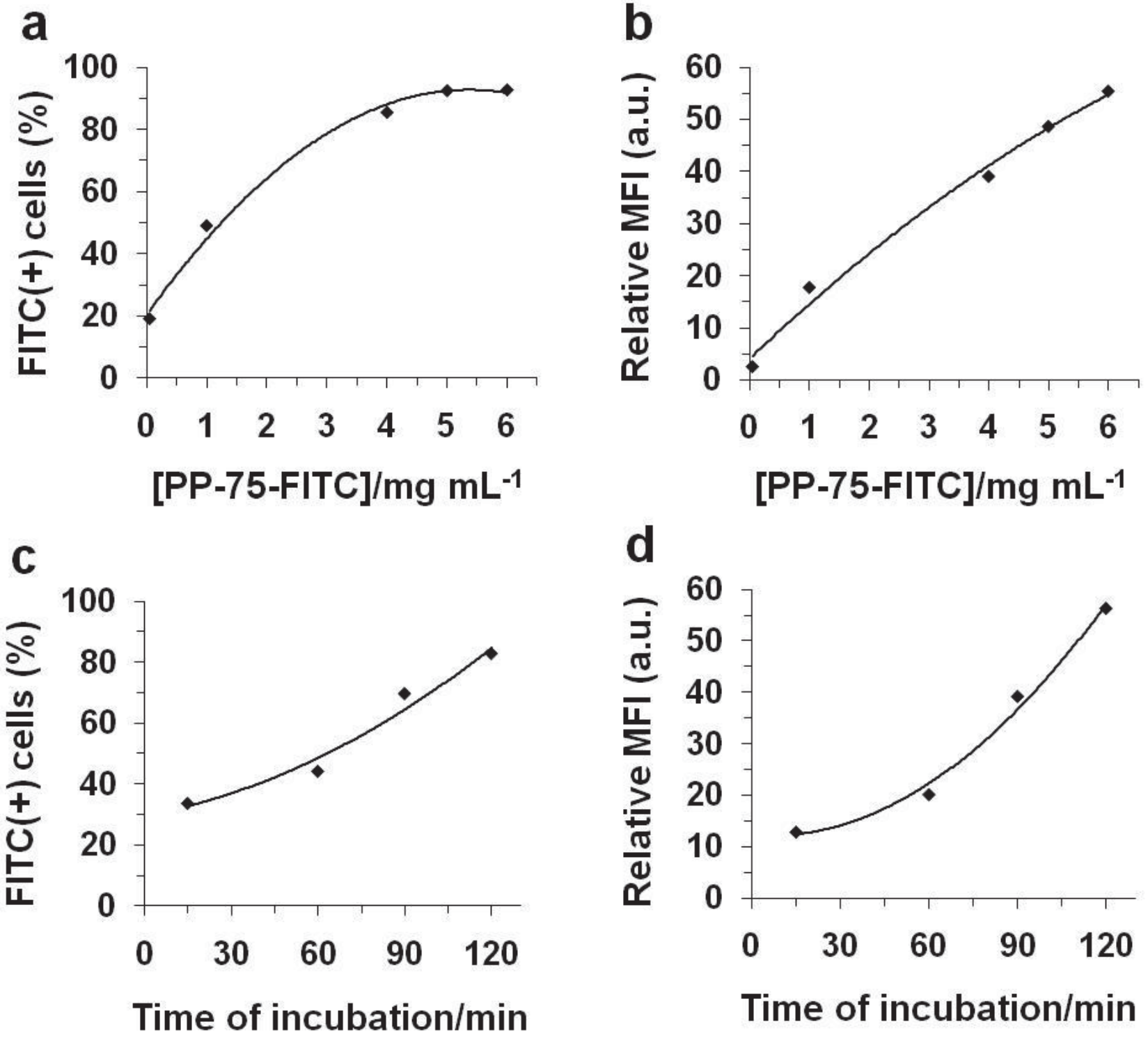


Figure 3


a
FITC(+) cells (%)
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[PP-75-FITC]/mg mL-1
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Relative MFI (a.u.)
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Figure 4

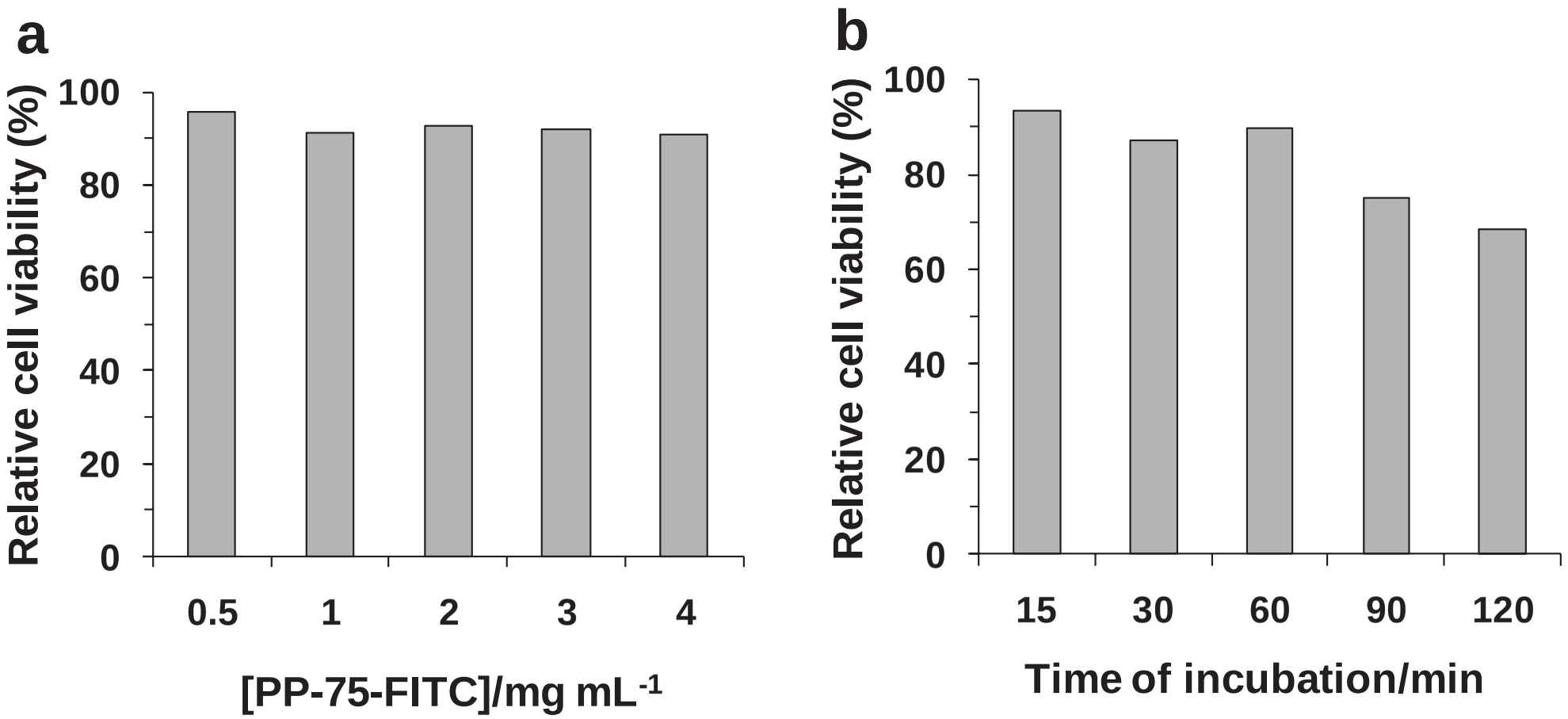

a
Relative cell viability (%)
100
80
60
40
20
0
0.5
1
2
3
4
[PP-75-FITC]/mg mL-1
b
Relative cell viability (%)
100
80
60
40
20
0
15
30
60
90
120
Time of incubation/min

Figure 5

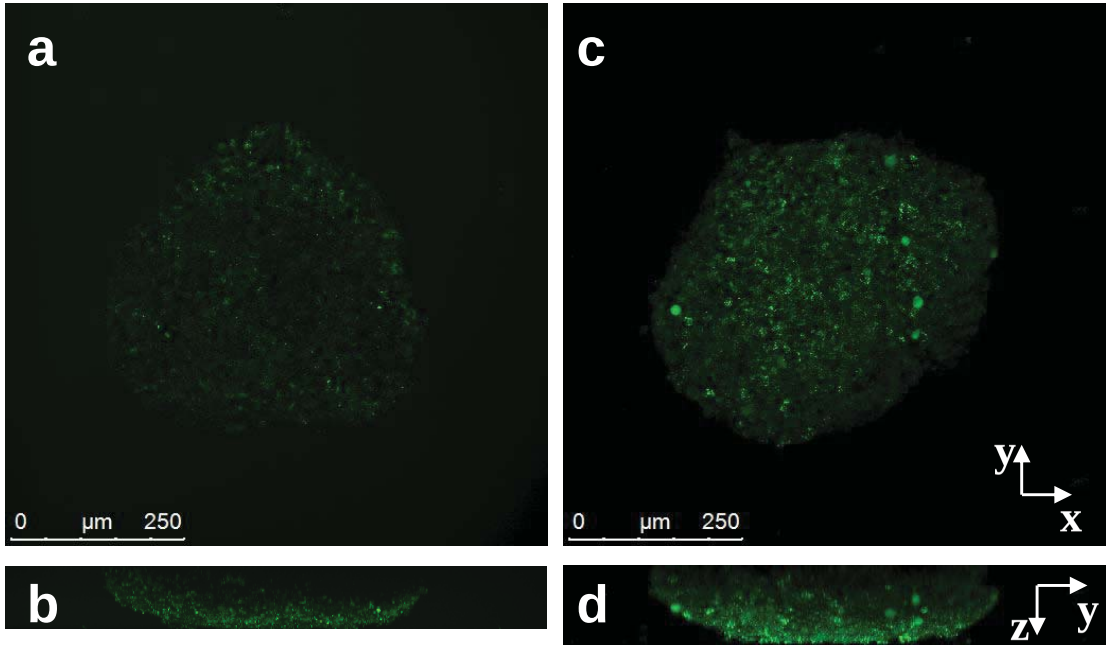
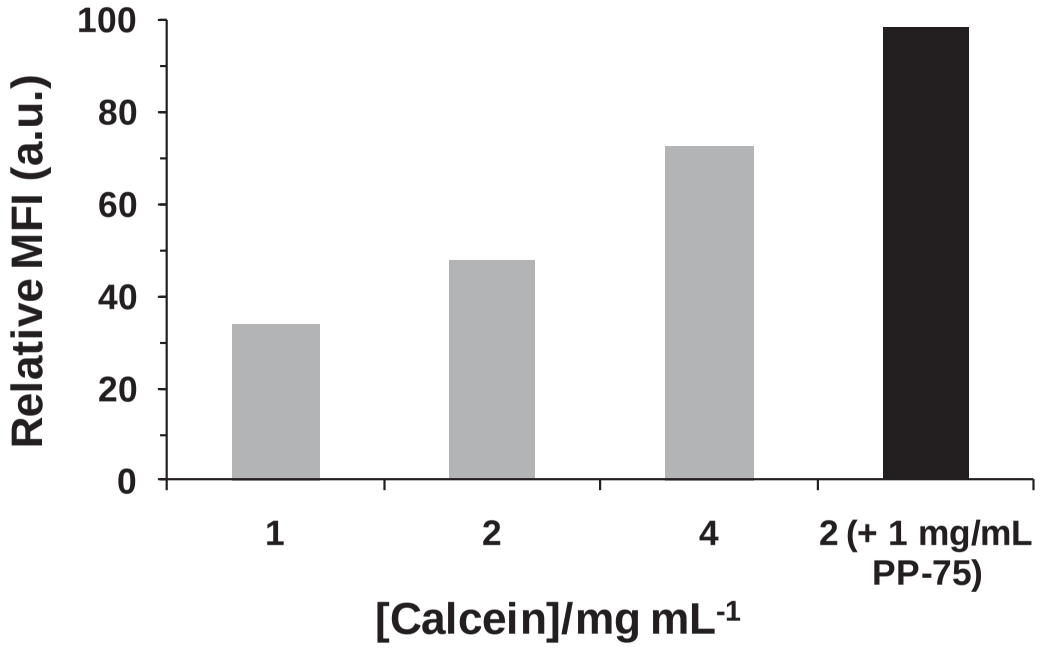


Figure 6


Relative MFI (a.u.)
100
80
60
40
20
0
1
2
4
2 (+ 1 mg/mL PP-75)
[Calcein]/mg mL-1

Supplementary Data

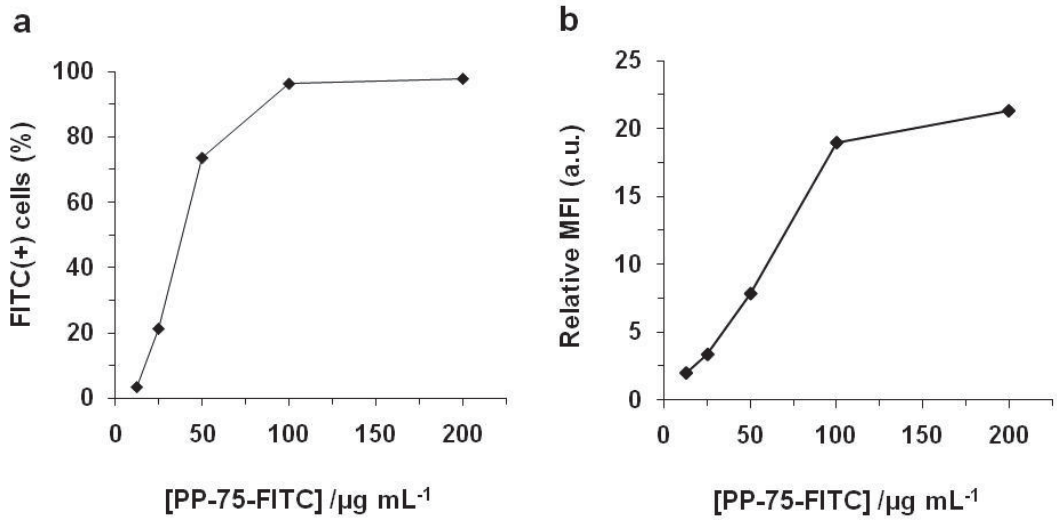


Fig. S1 Flow cytometric analysis of cellular uptake of PP-75-FITC in HeLa cell monolayers. Samples were treated with PP-75-FITC for 1 h and tested after 3.5 h further incubation. (a) Proportion of FITC-positive cells. (b) Relative mean fluorescence intensity (MFI) of cell population expressed in arbitrary units (a.u.). Relative MFI = ($MFI_{sample}/MFI_{control}$).