J Fluoresc (2008) 18:155–161
DOI 10.1007/s10895-007-0254-5

ORIGINAL PAPER

YOYO as a Dye to Track Penetration of LK15 DNA Complexes in Spheroids: Use and Limits

Heather AD Perry · Amer F. Alhaj Saleh · Harmesh Aojula · Alain Pluen

Received: 24 May 2007 / Accepted: 4 September 2007 / Published online: 24 October 2007

Abstract To elucidate the reasons underlying the poor penetration of non-viral vectors in tissues, relating transport properties to physico-chemical parameters of vectors may be crucial. These properties can be influenced by the presence of multiples labels that are used. Therefore utilizing a vector with minimum of labels preferably not more than one is important to studying penetration in tissues. The cell impermeant bisintercalating dye YOYO-1 was found suitable to both monitor the formation of complexes between DNA and an amphipathic peptide LK15 and, to track their penetration in HCT116 spheroids by confocal microscopy. The results revealed a limited decrease of fluorescence ascribed to the high affinity of YOYO-1 to bind DNA. The residual fluorescence of complexes can be exploited to monitor penetration into spheroids, after correction for YOYO-1 attenuation, and to revealing hyaluronidase-induced reduced binding. Hence high affinity dyes such as YOYO-1 with inefficiently quenched fluorescence may be important to establish a relation between novel medicines characteristics and penetration in tissues.

Keywords Non-viral gene delivery · Penetration · Spheroid · YOYO-1 · Confocal microscopy.

H. A. Perry · A. F. Alhaj Saleh · H. Aojula · A. Pluen (✉)
Drug Delivery group, School of Pharmacy and Pharmaceutical Sciences, University of Manchester, Oxford Road, Manchester M13 9PT, UK
e-mail: alain.pluen@manchester.ac.uk

Introduction

Non viral gene delivery systems (NVGDS) clinical efficacy which depends on sufficient delivery of complexes to target tissues, is limited by the poor ability of polyplexes to overcome physiological barriers, including the cell membrane, intracellular compartments and the interstitium [1]. Consequently being able to track these systems to find out their fate is essential in order to rationally design efficient systems.

Fluorescence techniques offer possibilities of quantitative characterisation of transport but their experimental conditions impose limitations. For instance, fluorescence recovery after photobleaching (FRAP) is destructive and requires a homogenous distribution of fluorescence—a difficult commitment for NVGDS due to low number of particles while the use of fluorescence correlation spectroscopy (FCS) may be limited due to the large NVGDS sizes compared to the width of the laser and FCS application to tissues remains complex though possible [2]. Therefore a simpler approach to observe drugs penetration with confocal microscopy as described by Kostarelos et al. [3] is worth considering. Indeed this method corrects image stacks by accounting for attenuation in tissues to provide more accurate penetration profiles. Although penetration is important, characterisation of the complex formation and their main physico-chemical parameters such as size and charge is also necessary to quantify transport. In fact, complex formation has largely been observed using the so-called ethidium bromide (or any other dye that may be excluded during the competition) assay [4]. The main disadvantages of such dyes are their exclusion from DNA binding site, high residual fluorescence when unbound to DNA and toxicity. Additionally, the resulting complexes are not suitable for

transport studies due to the presence of unbound dye causing high background against a low fluorescence signal of the complexes. Consequently, to relate complex formation, physico-chemical properties of the complex and tracking in tissues, it would be advantageous to use the same NVGDS. We show here that the bisintercalant dye YOYO-1 which has been used previously to monitor uptake of complexes in cells [5] and is apparently quenched but not excluded, could also serve to observe the penetration of the resulting complexes by confocal microscopy in 3D cellular network. YOYO-1 main features include a high binding affinity (3–4 higher than ethidium bromide [6]), an extremely low fluorescence level when unbound to DNA (1,000 times less), a decrease of fluorescence intensity during fluorescence assay/complexation thought to be related to quenching of the dye, not exclusion [7], being impermeant should limit uptake of possible free YOYO-1 by live cells and, finally, stability with pH rendering this dye more attractive than a Fluorescein based NVGDS which may be affected by pH changes in tissues.

To demonstrate the potential use of YOYO-1 as a suitable dye, we considered using a short ideal cationic amphipathic peptide based on lysine (K) and leucine (L) residues, LK15, primarily developed as antimicrobial agent and the plasmid pEGFPLuc. LK15 has been shown to form complexes with DNA by ethidium bromide displacement assay [4]. To assess the ability of the resulting complexes to be observed in tissues, we used multicellular tumour spheroids (MCTS) prepared with a human colorectal carcinoma cell line, HCT116. MCTS have been widely used in radiation oncology and are considered as a valid model for avascular tumours or metastases [8]. As tumour mimicking systems, MCTS have a complex spatial arrangement and contain certain levels of extracellular matrix, ECM, which has been shown to hinder penetration of macromolecular systems [9]. Consequently MCTS can be used to evaluate the influence of the barrier posed by ECM.

In this work complexes were formed between plasmid DNA pEGPLuc and LK15 peptide (polyplexes) in presence of YOYO-1. Their size and charge properties were characterised and concentration profiles determined in MCTS using confocal image stacks. Finally, we assessed the influence of glycosaminoglycans on NVGDS penetration by pre-incubating spheroids with BTH which cleaves glycosilic bonds of hyaluronic acid, chondroitin and chondroitin sulfate, upon which, NVGDVs may penetrate further into the spheroid. The results reveal that residual fluorescence of the YOYO-1 can be advantageously used to monitor penetration of NVGDS without recourse to any further labelling.

Materials and methods

Materials

YOYO-1 and YO-PRO-1 were purchased from Invitrogen (Cambridge, UK). The 6.4 kbp plasmid, pEGFPLuc, was purchased from Clontech (California, USA) and propagated in *E. coli* DH5α strain and purified with a Qiagen EndoFree Maxi Prep Kit (Crawley, UK) as per manufacturer's instructions. Deoxyrionucleic acid triphosphate was purchased from New England Biolabs (US).

Methods

Peptide synthesis

LK15 peptide (KLL KLL LKL LLK LLK) was synthesised by conventional solid-phase chemistry using Fmoc chemistry [10] using wang resin and Nα-protected amino acids purchased from Calbiochem Novabiochem (Beeston, UK). Following cleavage of the peptide with 95% trifluoroacetic acid in water the crude material was purified by reverse-phase HPLC using a semi-preparative C_4 vydac column. The purity was confirmed by reverse phase analytical HPLC and characterisation made by MALDI-MS.

Preparation of DNA/polycation complexes

Prior to use DNA (50 μg/ml) was labelled with YOYO-1 (10^{-4}M stock solution prepared in DMSO) at 1:50 and 1:400 ratio of dye molecule to DNA basepair (d/bp) as previously described [11]. Complexes of YOYO-1 labelled DNA with peptides were prepared in 15 mM HEPES buffer, pH 7.4 and 150 mM NACl by drop-wise addition of polycation to YOYO-1 labelled DNA. The preparation was done at room temperature (final DNA concentration 10 μg/ml). The different concentrations of peptide used are provided in Table 1.

Fluorescence spectroscopy and microscopy Emission spectra of DNA-bound YOYO-1 were recorded on a Perkin Elmer LS50B fluorimeter with excitation wavelength set at 480 nm and emission collected at 515 nm. DNA condensation was calculated as $(FL_{compl}/FL_{NDNA}) \times 100$, where FL_{compl} and FL_{NDNA} were peak fluorescence emissions of complexed and Naked DNA, respectively. Samples on

Table 1 Amount of LK15 necessary to form LK15/DNA complexes

LK15/DNA ratio	1:1	3:1	5:1	7:1	10:1
[LK15], μM	8.07	24.2	40.4	56.5	80.7

slides were observed with a combi LSM510 Meta (Zeiss, Jena, Germany). The wavelengths used were 488 nm wavelength for YOYO-1, 543 nm for the DIC and the epifluorescent light with appropriate filters for DAPI/ Hoechst 33342. Pictures were taken using a 40×1.3NA DIC oil immersion objective for cell monolayers and a 20× 0.5 NA fod MCTS. Image stacks were then deconvolved using AutoDeblur (MediaCybernetics, UK).

Characterisation of the physico-chemical parameters Light scattering measurements were taken at 37°C with a Brookhaven BI 200S instrument (Brookhaven, New York, USA) with a vertically polarised incident light source of wavelength 488 nm from a 2W argon ion laser (Coherent Innova 90, California, USA). Scattered light was collected through a Brookhaven BI 9000 AT digital correlator and correlation functions were analysed by the CONTIN method. Samples were prepared in triplicate and data collected over 2 minutes for each sample. Scattered light was filtered to remove fluorescence contribution to signal. To determine the zeta potential, samples were analysed in triplicate on a Malvern Zetasizer 3000HS (Malvern, Worcestershire, UK) after calibration with +50 mV (±5 mV) standards (Malvern, Worcestershire, UK).

Cell culture and spheroids formation

HCT116 and NIH3T6 cells were grown in DMEM containing 10% (*v*/*v*) FCS (Biowest, East Sussex, UK), 2 mM L-glutamine, 100 IU/ml penicillin and 2 mg/ml streptomycin. Cells were trypsinised at ~70% confluence with 0.5% (*w*/*v*) trypsin-EDTA in PBS. Spheroids were formed using liquid overlay technique i.e. cell suspensions were added to 1.5% *w*/*v* agarose gels containing plates, in presence of the above medium and incubated at 37°C, 5% CO_2 in relative humidity.

Hyaluronidase treatment

Based on a study by Brekken and Davies [12], spheroids were treated with 10^{-4}% *w*/*v* BTH in 150 mMPBS or PBS alone as this concentration was found to be non-toxic to cell monolayers for one hour before transfection as described below.

Transfection studies

Cells were seeded at 2.0×10^5 cells/well in six-well cluster plates and allowed to reach ~60% confluence. For confocal laser scanning microscopy (CLSM), cells were grown on acid washed coverslips and placed in the cluster plates. Growth media was aspirated, cells rinsed with PBS and pre-incubated with 1 ml OptiMEM, BTH (Bovine Testes Hyaluronidase, Sigma) and phosphate buffered saline (PBS) or PBS alone. Pre-incubation was followed by a period of recovery in fresh OptiMEM. Cells or spheroids were then transfected with 1 μg complexed DNA in 1 ml of warmed OptiMEM for 4 h. Samples for CLSM were rinsed and nuclei stained with 20 μM Hoeschst 33342 in DMEM and coverslips mounted onto glass slides with Dako Fluorescent Mounting Media.

Luciferase assay

Twenty-four hours after transfection, luciferase expression was measured with a Promega Luciferase Assay Kit (number E1501) as per manufacturer's instructions. Cells were lysed and debris cleared by centrifugation at 12,000×*g* for 10 s and 20 μl aliquots made in triplicate into opaque 96-well cluster plates. Activity was measured using a Fluostar Optima luminometer from BMG Labtech (Aylesbury, UK) reader as relative light units (RLU) emitted over 10 s after a 2 s delay following automated injection of 100 μl luciferase assay substrate. Activity was normalised for protein content, determined by the BCA protein assay.

Results and discussion

Fluorescence assay and formation of complexes

Fluorescence assay is one of the most common methods to demonstrate complexes formation between DNA and a ligand. In these assays the decrease of fluorescence reflects the competition between the ligand and the dye to access the negative charges of DNA. Figure 1 presents the variation of the fluorescence intensity as a function of LK15 concentration (see Table 1 for conversion in LK15/ DNA ratios) observed after 30 min of incubation of the samples at different DNA:dye ratios. Two different dyes were used. As expected the addition of peptide leads to a decrease of fluorescence initially suggesting YOYO-1 and LK15 compete to access to the negative charges of DNA. Interestingly the high remaining fluorescence level (~60%) of YOYO-1 labelled complexes even after a large variation of the number of YOYO-1 dyes added to DNA (1:400 or 1:50) may indicate a poor binding affinity of LK15. A change of fluorescent dye from a bisintercalator YOYO-1 to an intercalator YOPRO-1 which has a lower binding affinity than YOYO-1 and may be excluded or quenched [13] leads to a large decrease of the fluorescence intensity at a 1:50 dye/DNA base pairs ratio (80% compared to

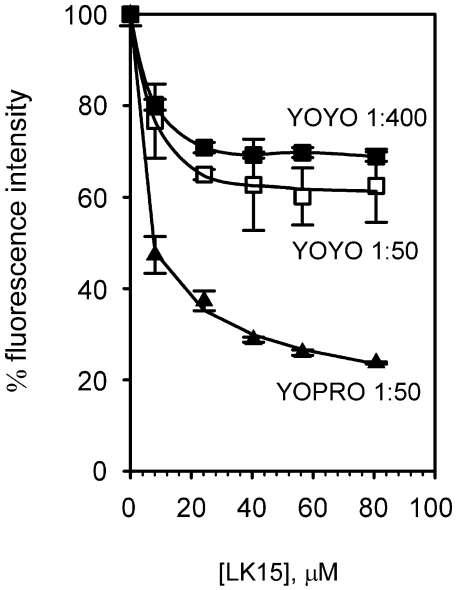

Fig. 1 YOYO-1 fluorescence assay using the plasmid DNA pEFPLuc at a concentration of 10 μg/ml providing the variation of relative fluorescence for each polymer concentration of LK15. Effect of the number of YOYO-1 dye per DNA base pairs (dye/bp) on the reduction of fluorescence: 1:400 (*filled squares*) and 1:50 (*open squares*) as well as effect of YOPRO-1 (1:50) (*closed triangles*), all at [NaCl]=150 mM and 15 mM HEPES. Each point is the result of three separate experiments in triplicate

39%). This large reduction of fluorescence is close to the level of dye displacement observed by Dufourcq et al. [4] with ethidium bromide or KALA another amphipathic peptide [14]. Hence LK15 can compete with intercalating dyes but experiences apparent difficulties in presence of YOYO-1. Conversely, YOYO-1 intrinsic properties may be responsible for the poor decrease of fluorescence intensity at high peptide:DNA ratios. Indeed Krishnamoorthy et al. [7] have shown that YOYO-1 is not excluded but quenched during this assay due to the formation of H-type dimers and, a d/bp ratio of 1:100 or above would be required to form enough dimers to reduce fluorescence. Interestingly, our results are in agreement with these predictions as a decrease of fluorescence is observed for 1:400 YOYO-1/DNA ratio. Above this possible threshold, increasing the dye concentration has little effect on the fluorescence decrease. Finally, the high residual fluorescence intensity of YOYO-1 based systems suggests that these complexes should be observed not only in solution but also in biological samples.

Characterisation of the complexes

Uptake by cells and ability to penetrate tissues have been found to depend on the charge of the non viral gene delivery system and its size [15]. To further improve the relevance of charge and size to the problem of tissue penetration, measurements were carried out in OptiMEM. In fact, size and charge were determined for three ratios only (1:1, 5:1 and 10:1). Figure 2 reveals a lack of significant variation of complexes' size (hydrodynamic diameter) at these ratios (about 80 nm). Additionally Fig. 2 provides the variation of the zeta potential, ζ, of LK15 based polyplexes for each peptide:DNA ratio. This potential varies from negative at 1:1 ratio (−17 mV) to positive at 5:1 and 10:1 ratios (13 and 17 mV respectively) suggesting complex formation at 5:1 and 10:1 ratios. Thus, DNA/LK15/YOYO-1 complexes are relatively small and positively charged at these ratios. As OptiMEM does not seem to influence complexe sizes [16], these results are then in good agreement with literature as similar sizes were observed with other cationic α-helical peptides that are based on 'LK' sequence with an added tryptophan (W) residue [17] or KW sequences only [18].

Visualisation of complexes in cell monolayers

Having established that complexes remain fluorescent, we were able to follow the internalisation of polyplexes in HCT 116 cells. Punctuate fluorescent spots (green colour) can be observed as shown in Fig. 3a,b indicative of polyplexes presence in or on BTH negative and positive HCT 116 cells as observed by CLSM. Thus confirming as expected [5] YOYO-1 labelled complexes can be observed in cells.

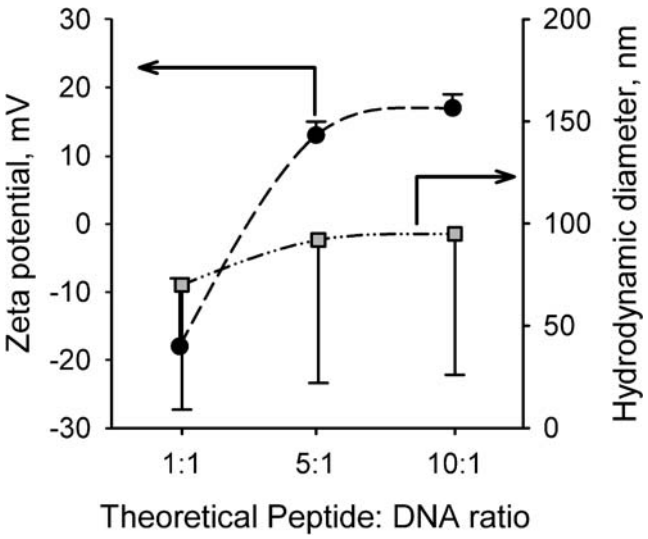

Fig. 2 Characterisation of the hydrodynamic radius (*grey squares*) and zeta potential (*black circles*) of LK15-based complexes at ratios 1:1, 5:1 and 10:1. 1 YOYO every 50 DNA bp, 25 μg complexed DNA diluted to 10 μg/ml in OptiMEM. Mean of triplicates, *error bar*=1 SD

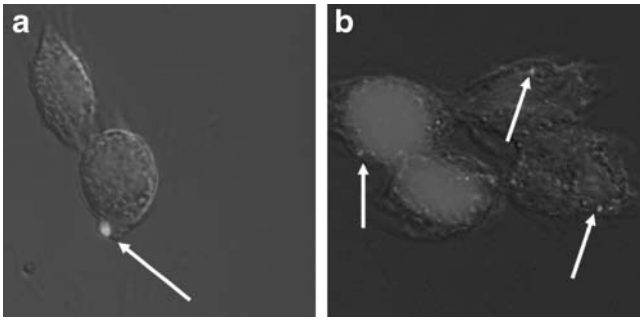

Fig. 3 Visualisation of complexes 5:1 in BTH negative and positive HCT 116 cells after 1 h in **a** untreated cells and **b** BTH treated cells. Live cells were grown onto slides with Dako fluorescent mounting media. Nuclei are stained with DAPI and *light grey spots* indicates YOYO-1 stained complexes. Imaged using 40×1.3NA Oil immersion objective

Transfection efficiency

As our plasmid encoded for EGFP, it was important to determine whether our complexes were efficient at delivering genetic information and whether GFP fluorescence should be accounted for after four hours when determining fluorescence profiles in spheroids. Consequently, transfection was assessed on HCT116 cells monolayers by luciferase assay 48 h after incubation for ratios 5:1 and 10:1 (only) and compared to controls. Luminescence (RLU) was chosen as more sensitive to monitor transfection than flow cytometry (GFP expression). Figure 4 indicates that the luminescence signal is very low and no significant variation of the RLU was observed between the LK15 based polyplexes (ratios 5:1 and 10:1) all of which produce approximately two orders of magnitude higher gene expression than pEGFPLuc alone. As low luminescence levels were observed 48 hours after incubation, it is unlikely that transfection i.e. GFP amount, will be high four hours after incubation and, thus, significantly affect the evaluation of complexes penetration by confocal microscopy. Consequently, during our assessment of complexes penetration in 3D cellular models, we will assume that inside the spheroid, fluorescence detected originates from YOYO-1 not from GFP expressed after transfection.

Penetration of HCT 116spheroids

The final objective of the study was to find out whether the LK15/YOYO-1/DNA complexes could be detected in MCTS, measure their penetration and whether or not pre-incubation by remodelling enzyme BTH would affect penetration and binding of complexes in MCTS. MCTS incubated with either 5:1 or 10:1 polyplexes for four hours were imaged by confocal microscopy. Figure 5 illustrates the ability of complexes to penetrate tumour spheroids: while fluorescent spots are observed on the first layers of the spheroids (10–30 μm deep), further sectioning into MCTS only indicates fluorescence in the outer rim of the spheroid (60–80 μm). This phenomenon suggesting more limited penetration than the reality is due to signal attenuation. In the early nineties, Wartenberg and Acker [19] devised an approach in which the observed decrease of fluorescence, $I(x)$, is related to the effective decrease of fluorescence (decrease of fluorescent dyes), I_0, by the following expression:

$$I(x) = I_0 \exp(-ar) \quad (1)$$

Where a is a parameter function of the specific attenuation of the dye and its concentration, and r is the radial distance from the edge of the MCTS.

To evaluate the penetration of the polyplexes after 4 h, fluorescence image stacks of spheroids were created. Figure 6 shows the profiles of relative fluorescence determined in the centre of the spheroid (area approx. five cells) for the 5:1 and 10:1 complexes pre or non-pre-treated with hyaluronidase. Raw data (Fig. 6a) confirm the influence of signal attenuation and indicate additionally similar accumulation levels at 20 μm for 5:1 and 10:1 complexes when only exposed to PBS (dark symbols). It is noteworthy that (1) a marked peak is observed at approx. 20 μm corresponding to the first layers of cells exhibiting the highest concentration of dyes at the interface MCTS/solution. (2) when the spheroids were treated with BTH (10^{-4}% *w*/*v*), the level of fluorescence intensity decreased significantly for both complexes especially 10:1 complexes

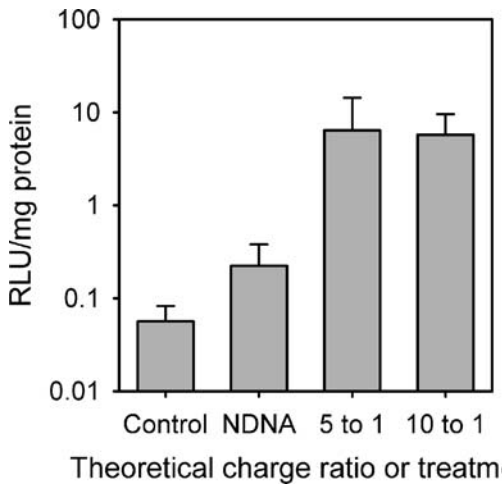

Fig. 4 Transfection of HCT116 cells monolayers by 5:1 and 10:1 LK15/DNA complexes (comparison to pEGFPLuc plasmid only and to PBS treatment alone—control). Three separate experiments in quadruplate, *bar*=1SD. Cells seeded at a concentration of 2.0×10^5 and transfected at 70% confluence in presence of 1 μg DNA or complexed DNA for 4 h

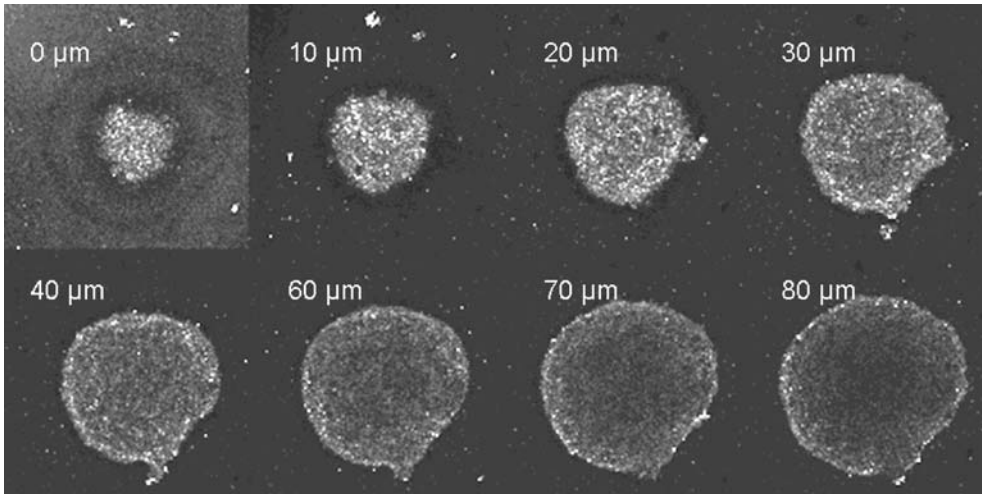

Fig. 5 Uptake and distribution of LK15/DNA complexes in HCT116 spheroids using confocal microscopy. The extent of localisation was determined following 4 h transfection with 5:1 complexes stained with YOYO-1 (ratio 1:50). The amount of DNA was 1 μg was used to form complexes. Images were obtained along the *z*-axis every 10 μm up to 100 μm

indicating that accumulation is altered in the outer rim of the spheroid by BTH treatment (reduced accumulation).

To correct for attenuation, the parameter, *a*, (see Eq. 1) was determined. As free YOYO-1 fluorescence is low, it had to be mixed with low molecular weight macromolecule to limit transport hindrance in tissues [9]: YOYO-1 was then mixed with deoxynucleotide triphosphate, dNTP, to increase the fluorescence levels. YOYO-1/dNTP complexes should have a relatively low molecular weight (approx. 1 kDa) and thus diffuse through the spheroids in less than 4 h. Hence the observed decay of fluorescence of YOYO-1/dNTP complexes should reflect the attenuation of YOYO-1 fluorescence intensity in the HCT116 spheroid rather than the hindrance to transport. Fluorescence profiles of dNTP/YOYO-1 after 4 h were fitted with a monoexponential decay and *a* determined ($a = 0.05 \pm 0.01\ \mu m^{-1}$)—data not shown. Corrected profiles using Eq. 1 (Fig. 6b) indicate a deep penetration of complexes in MCTS. Considering the maximum penetration as obtained when the level of fluorescence is still different from the cell background (noted control in Fig. 6), non BTH treated 5:1 and 10:1 complexes seem to have penetrated approx. 70 μm in 4 h. Based on this, a reasonable diffusion coefficient ($\sim 3.5 \times 10^{-9} cm^2 \cdot s^{-1}$) was estimated not too dissimilar to diffusion coefficients of 80 nm radius liposomes measured in low collagen content tumour ($5 - 6 \times 10^{-9} cm^2 \cdot s^{-1}$) [9].

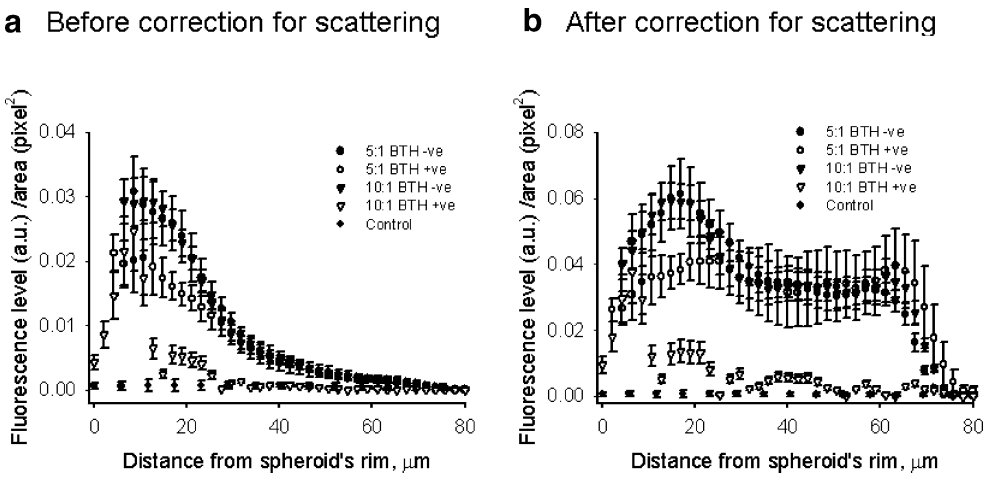

Fig. 6 Effect of charge ratio and BTH on penetration in HCT 116 spheroids. Comparative fluorescence intensity (arbitrary units/measurement area in pixels^2) profiles relative to the distance from the spheroid rim to the centre for 5:1 and 10:1 LK15/DNA complexes in presence or absence of BTH at 10^{-4}% *w*/*v*. Control is the fluorescence background of HCT116 spheroids treated with PBS only. **a** Raw data. **b** Corrected profiles. $N=3$

Although measurements were not done as time course it reveals the feasibility of the method and in the present case may underestimate the true diffusion coefficient and penetration distance. Finally the method allows for determining the effect of BTH (10^{-4}% *w*/*v*) on transport in MCTS as the level of fluorescence intensity decreased significantly for 10:1 complexes suggesting BTH dramatically affects the accumulation of the dye in tissues.

Conclusion

This present study demonstrates that a widely used dye, YOYO-1, with fluorescence quenching properties and high affinity binding may be used not only to observe the formation of a complex but also to assess their penetration in three dimensions cellular assemblies such as MCTS and to provide information on binding and diffusion comparable to other methods. This approach has the major advantage of reducing effects of modifications of the systems and hence, errors associated with the traditional approaches where different labels are used. It should be noted though that this approach like any the other approaches is not completely free of the influence on the polyplexes transfection efficiency. It is quite conceivable that the presence of YOYO-1 affects the complex and impairs its intracellular trafficking. Consequently using YOYO-1 or any similar fluorescent dye is of interest for evaluating complexes penetration/transport to determine the fate of poorly efficient non viral gene delivery systems, but not their ability to transfect cells. Finally, as YOYO-1 is a stable fluorescent dye, one can imagine that fluorescence correlation spectroscopy could be used to determine the size of the complexes as well as the kinetics of the formation.

Acknowledgments HP was supported by a BBSRC studentship. Prof IJ Stratford's Experimental Oncology group, Prof L Kunz-Schughart and Prof D Attwood for light scattering and Eva Durant School of Paper Science, UMIST for zetasizer. Mrs N Ning for spheroids culture and British Council (ARC 1226) for support.

References

1. Jain RK (2001) Delivery of molecular and cellular medicine to solid tumors. Adv Drug Deliv Rev 46(1–3):149–168
2. Alexandrakis G, Brown EB, Tong RT, McKee TD, Campbell RB, Boucher Y, Jain RK (2004) Two-photon fluorescence correlation microscopy reveals the two-phase nature of transport in tumors. Nat Med 10(2):203–207
3. Kostarelos K, Emfietzoglou D, Papakostas A, Yang WH, Ballangrud A, Sgouros G (2004) Binding and interstitial penetration of liposomes within avascular tumor spheroids. Int J Cancer 112(4):713–721
4. Dufourcq J, Neri W, Henry-Toulme N (1998) Molecular assembling of DNA with amphipathic peptides. Febs Lett 421(1):7–11
5. Kopatz I, Remy JS, Behr JP (2004) A model for non-viral gene delivery: through syndecan adhesion molecules and powered by actin. J Gene Med 6(7):769–776
6. Gurrieri S, Wells KS, Johnson ID, Bustamante C (1997) Direct visualization of individual DNA molecules by fluorescence microscopy: Characterization of the factors affecting signal/background and optimization of imaging conditions using YOYO. Anal Biochem 249(1):44–53
7. Krishnamoorthy G, Duportail G, Mely Y (2002) Structure and dynamics of condensed DNA probed by 1,1 '-(4,4,8,8-tetramethyl-4,8-diazaundecamethylene)bis[4-[[3-methylbenz-1,3 -oxazol-2-yl]methylidine]-1,4-dihydroquinolinium] tetraiodide fluorescence. Biochemistry 41(51):15277–15287
8. Kunz-Schughart LA (1999) Multicellular tumor spheroids: intermediates between monolayer culture and in vivo tumor. Cell Biol Int 23(3):157–161
9. Pluen A, Boucher Y, Ramanujan S, McKee TD, Gohongi T, di Tomaso E, Brown EB, Izumi Y, Campbell RB, Berk DA, Jain RK (2001) Role of tumor-host interactions in interstitial diffusion of macromolecules: Cranial vs. subcutaneous tumors. Proc Natl Acad Sci USA 98(8):4628–4633
10. Atherton E, Sheppard RC (1989) Solid phase peptide synthesis: a practical approach. IRL Press, Oxford
11. Carlsson C, Larsson A, Jonsson M (1996) Influence of optical probing with YOYO on the electrophoretic behavior of the DNA molecule. Electrophoresis 17(4):642–651
12. Brekken C, Davies CD (1998) Hyaluronidase reduces the interstitial fluid pressure in solid tumours in a non-linear concentration-dependent manner. Cancer Lett 131(1):65–70
13. Murayama Y, Sano M (2005) Exchange of counterions in DNA condensation. Biopolymers 77(6):354–360
14. Wyman TB, Nicol F, Zelphati O, Scaria PV, Plank C, Szoka FC (1997) Design, synthesis, and characterization of a cationic peptide that binds to nucleic acids and permeabilizes bilayers. Biochemistry 36(10):3008–3017
15. Pouton CW, Seymour LW (2001) Key issues in non-viral gene delivery. Adv Drug Deliv Rev 46(1–3):187–203
16. Pouton CW, Lucas P, Thomas BJ, Uduehi AN, Milroy DA, Moss SH (1998) Polycation-DNA complexes for gene delivery: a comparison of the biopharmaceutical properties of cationic polypeptides and cationic lipids. J Control Release 53(1–3):289–299
17. Niidome T, Takaji K, Urakawa M, Ohmori N, Wada A, Hirayama T, Aoyagi H (1999) Chain length of cationic alpha-helical peptide sufficient for gene delivery into cells. Bioconjug Chem 10 (5):773–780
18. Tokunaga M, Hazemoto N, Yotsuyanagi T (2004) Effect of oligopeptides on gene expression: comparison of DNA/peptide and DNA/peptide/liposome complexes. Int J Pharm 269(1):71–80
19. Wartenberg M, Acker H (1995) Quantitative recording of vitality patterns in living multicellular spheroids by confocal microscopy. Micron 26(5):395–404