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pH-sensitive intracellular photoluminescence of carbon nanotube–fluorescein conjugates in human ovarian cancer cells

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Abstract
To add to the understanding of the properties of functionalized carbon nanotubes in biological applications, we report a monotonic pH sensitivity of the intracellular fluorescence emission of single-walled carbon nanotube–fluorescein carbazide (SWCNT–FC) conjugates in human ovarian cancer cells. Light-stimulated intracellular hydrolysis of the amide linkage and localized intracellular pH changes are proposed as mechanisms. SWCNT–FC conjugates may serve as intracellular pH sensors.

(Some figures in this article are in colour only in the electronic version)

1. Introduction

Biocompatibility and the availability of accessible methods for functionalization with bio-active molecules make single-walled carbon nanotubes (SWCNTs) promising candidates for intracellular delivery agents and for other applications in bio-sensing and biomedicine [1–4]. Many studies have focused on the use of SWCNTs as vehicles for direct delivery of anti-cancer drugs across the cell membrane of cancer cells [5, 6]. Utilization of the optical and electronic properties of carbon nanotubes for intracellular sensing has been less explored [7, 8]. Heller and colleagues found that DNA-encapsulated nanotubes can be used to sense intracellular cationic concentrations. After the addition of divalent cations such as Hg^{2+}, the transition of DNA secondary structure from a B to Z conformation modulated the dielectric environment of SWCNTs and decreased their near-IR emission energy, thus yielding observable characteristic shifts in the SWCNT luminescence spectrum [9]. Intracellular pH (pH_i) is a key parameter in most biochemical and biological processes [10]. Conventional methods for measuring pH_i include the use of radioactively labeled weak organic acids and bases [11, 12], nuclear magnetic resonance spectroscopy (NMR) [13, 14], and fluorescent dyes [15]. However, as many disease states are associated with abnormal chemical environments at a subcellular level; new probes are needed that measure pH in localized environments inside living cells in real time. The development of nanoparticle-based fluorescent probes represents a new trend that offers the advantages of localized and continuous pH_i monitoring with high time resolution and sensitivity [16–18]. Although SWCNTs are widely used as intracellular carriers, little has been reported about utilizing them as pH_i probes. In this work, we have covalently linked carbon nanotubes to fluorescein 5-thiosemicarbazide (SWCNT–FC) and evaluated the response of the conjugate as a probe for intracellular processes involving pH_i changes in

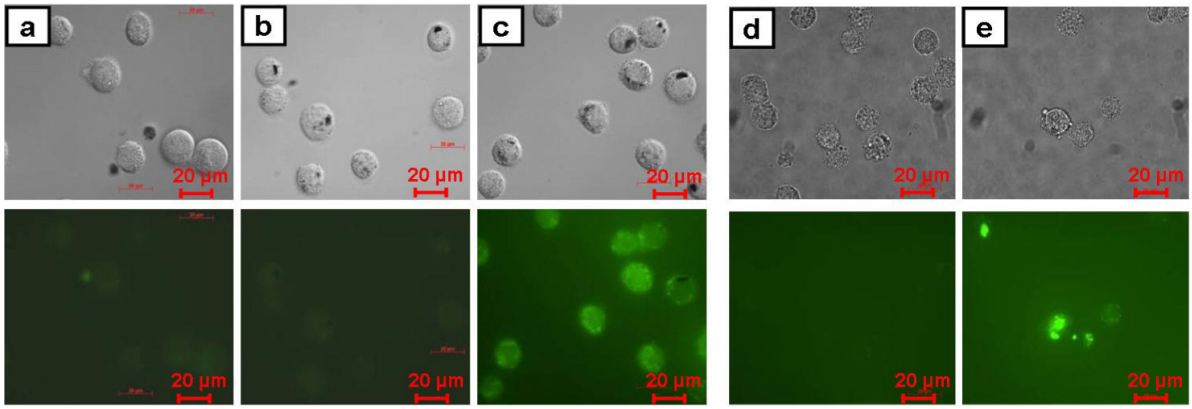

Figure 1. Differential interference contrast (DIC) (upper panels) and fluorescence (lower panels) images of SKOV-3 cells incubated in RPMI 1640 for 17 h with (a) FC, (b) a mixture of SWCNTs and FC, and (c) SWCNT–FC conjugates, and for 1 h with SWCNT–FC conjugates at 4 °C (d) and 37 °C (e).

SKOV-3 human ovarian cancer (SKOV-3) cells. Fluorescence of SWCNT–FC conjugates in SKOV-3 cells was found to increase upon light stimulation. Two hypotheses are proposed to explain such observations: first, de-quenching of fluorescein fluorescence could result from light-stimulated intracellular hydrolysis of the amide linkage between SWCNTs and fluorescein molecules. Second, light-induced intracellular pH changes may cause fluorescence intensity variations of the SWCNT–FC conjugates, since fluorescein is a pH-sensitive dye. Either of the above-mentioned processes, or both, would produce a fluorescence intensity increase upon light stimulation. Our results demonstrate that nanosized conjugates like SWCNT–FC have the potential to be used as localized pH_i sensors and trackers in studies of uptake mechanisms, endocytotic degradation, and photo-induced phenomena in living cells.

2. Materials and methods

2.1. Synthesis of carbon nanotubes-fluorescein (SWCNT–FC) conjugates

HiPco SWCNTs (Carbon Nanotechnology Inc.) were shortened and carboxylated by acid reflux treatment [19–21] to yield highly functionalized nanotubes about 200 nm long with carboxylic acid groups around the open ends and at defect sites in the sidewalls. The mole per cent concentration of carbon atoms belonging to carboxyl groups in oxidized SWCNTs by this method has been reported to be between 3.6% and 8.0% [21]. Oxidized nanotubes were reacted with fluorescein-5-thiosemicarbazide (FC) (Sigma-Aldrich) and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (Sigma-Aldrich) to form covalent conjugates through an amide linkage. SWCNT–FC conjugates were then filtered, rinsed thoroughly to remove unreacted materials, and re-dispersed in Dulbecco's Phosphate Buffered Saline (PBS 1X) solution without calcium and magnesium salts (Irvine Scientific, Irvine, CA) to a SWCNTs concentration of 0.25 mg ml^{-1}.

2.2. Cell lines and fluorescence microscope

White-light and fluorescence microscopy were employed to evaluate the uptake of SWCNT–FC conjugates by SKOV-3 cells (ATCC, Manassas, VA) cultured in RPMI 1640 medium (Irvine Scientific, Irvine, CA) containing 10% heat-inactivated fetal bovine serum (FBS; Gibco, Carlsbad, CA), 2 mM L-glutamine (Gibco, Carlsbad, CA), 45 units ml^{-1} penicillin (Gibco, Carlsbad, CA), and 45 μg ml^{-1} streptomycin (Gibco, Carlsbad, CA). Cells were incubated with solutions of: FC [0.025 mg ml^{-1}], SWCNT [0.025 mg ml^{-1}] mixed with FC [0.025 mg ml^{-1}], and SWCNT–FC [0.025 mg ml^{-1}] conjugates for 17 h in RPMI 1640 culture medium. Cells were washed to remove extracellular nanotubes and dye molecules prior to fluorescence imaging. A Zeiss Axiovert 200 M fluorescence microscope (Carl ZeissMicroImaging, Inc., Thornwood, NY, USA) was used for imaging. Filter sets for SWCNT–FC 480 nm excitation and 540 nm emission, respectively.

3. Results

3.1. Internalization of SWCNT–FC

Figures 1(a)–(c) compare the FC uptake levels by SKOV-3 cells incubated with free FC, SWCNTs mixed with FC, and SWCNT–FC conjugates, respectively. Unlike their unbonded counterparts where the uptake is minimal, SWCNT–FC conjugates were able to readily cross the cell membrane and exhibit strong fluorescence from the cytoplasm and intracellular organelles. The SWCNT–FC conjugates entry mechanism was evaluated by comparing uptake at low (4 °C) and normal growth temperatures (37 °C). After 1 h, only cells incubated at 37 °C (figure 1(e)) show strong fluorescence due to internalized SWCNT–FC conjugates; no detectable emission was observed in cells incubated at 4 °C (figure 1(d)). This result is consistent with an energy-dependent endocytotic pathway as the SWCNT–FC conjugates uptake mechanism [22]. Figure 2(a) depicts a SEM image of fixed SKOV-3 cells incubated with SWCNT–FC conjugates. Interestingly, nanotube-like structures were found across the

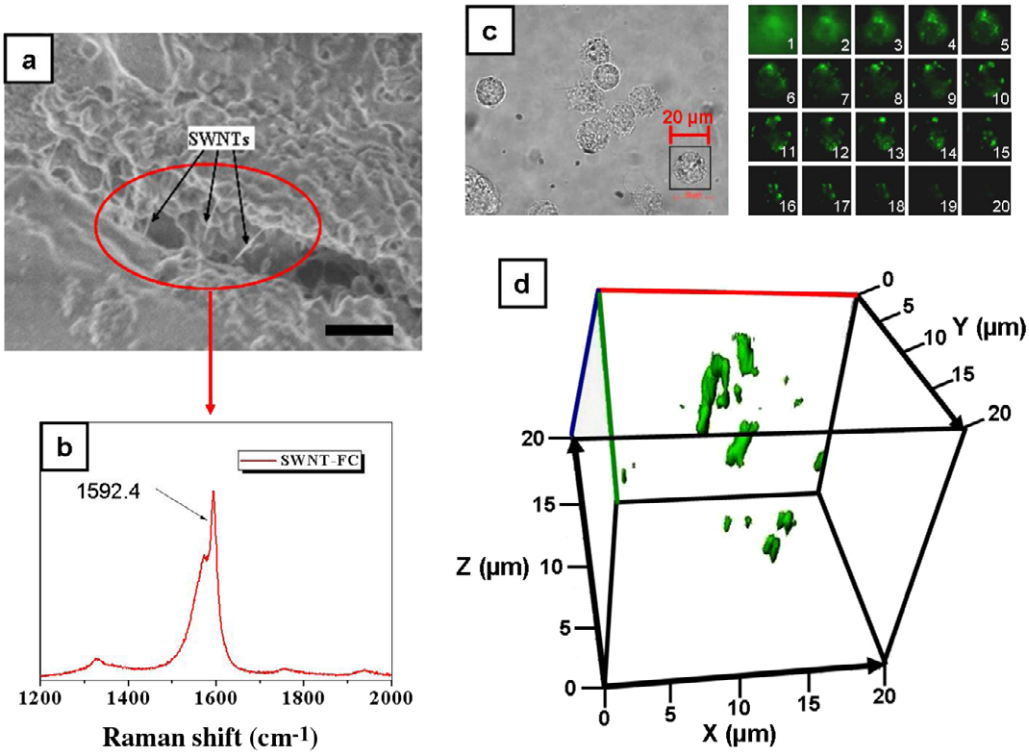

Figure 2. (a) SEM image of fixed intracellular SWCNT–FC SKOV-3 cells; (b) micro-Raman spectrum of intracellular SWCNTs showing a G-band peak at 1592.4 cm^{-1}; (c) left: optical microscopy image of SKOV-3 cell culture previously incubated with SWCNT–FC conjugates, right: fluorescence microscopy images taken at different focus planes in Z-direction from top to bottom. The interplane distance is 1 μm; (d) three-dimensional representation of the data collected in (c).

cell membrane. The micro-Raman spectrum of one of these structures reveals a strong G-band peak at 1592 cm^{-1}, which is characteristic of SWCNTs (figure 2(b)). In order to gather more information in regards to intracellular SWCNT–FC, the cell was chosen inside square on the left white-light image of figure 2(c). Fluorescence images were taken at different focal planes along the z-axis. The distance between each focal plane is 1 μm. The resulting 3D fluorescence image is shown in figure 2(d) with Zeiss AxioVision 3.1 imaging software. Vesicle-like intracellular fluorescence emission sources were found to be localized in the perinuclear region in the cytoplasm.

3.2. Photoluminescence of SWCNT–FC within cells

Interestingly, we observed that intracellular SWCNT–FC conjugates exhibit a fluorescence emission enhancement after light stimulation. SKOV-3 cells incubated with SWCNT–FC were exposed for 2.5 s to the 480 nm band-pass-filtered emission of a mercury lamp (source power density = 0.04 W m^{-2}), after which fluorescence microscopy images were taken at 0, 5 and 10 min (figure 3(a)). We integrated and averaged the fluorescence intensity of cells from each image in figure 3(a). Comparison of the averaged fluorescence intensity at 0 and 10 min reveals a 50% increase in fluorescence intensity (date were analyzed in figure 7). To further confirm that the intracellular fluorescence intensity increase was caused by light stimulation, we used a 63× objective to irradiate a small area 300 μm in diameter, and then we imaged a larger concentric area 1 mm in diameter with a 10× objective. Figure 3(b) clearly shows that there is a stronger fluorescence emission area which corresponds to the region irradiated with the 63× objective. The regions photo-excited later with the 10× objective show much lower fluorescent emission. This result demonstrates that exposing intracellular SWCNT–FC conjugates to light stimulation produces a strong increase in fluorescence intensity and suggests an intracellular photo-activated process that was sensed by the SWCNT–FC conjugates. To understand the nature of the observed photo-activation phenomenon, the time evolution of the intracellular SWCNT–FC fluorescence intensity after being photo-activated was further studied. We first photo-exposed SKOV-3 cells with intracellular SWCNT–FC conjugates for 2.5 s, then the cells were placed in fresh growth medium for 36 h. After incubation, cells were separated into two samples, washed thoroughly and photo-exposed again for either 2.5 s (figure 3(c)) or 1.5 s (figure 3(d)). Note that figures 3(a) and (c) reveal that although the fluorescence intensity of SWCNT–FC conjugates increases with time in all areas within the cells, it becomes stronger within intracellular organelles than in the cytoplasm. This behavior may be attributed to the activation of a photo-stimulated process that affects the endosomal membranes. Disruption/perturbation of the endosomal membranes may allow an increase in the endosomal pH and/or facilitate the diffusion of the fluorescence emitters from organelles to the cytoplasm, where they find a higher pH and therefore exhibit stronger fluorescence [23]. Comparisons of figures 3(c) and (d) reveal decreased fluorescence intensity when the photo-activation time was reduced from 2.5 to 1.5 s (110% and 60%,

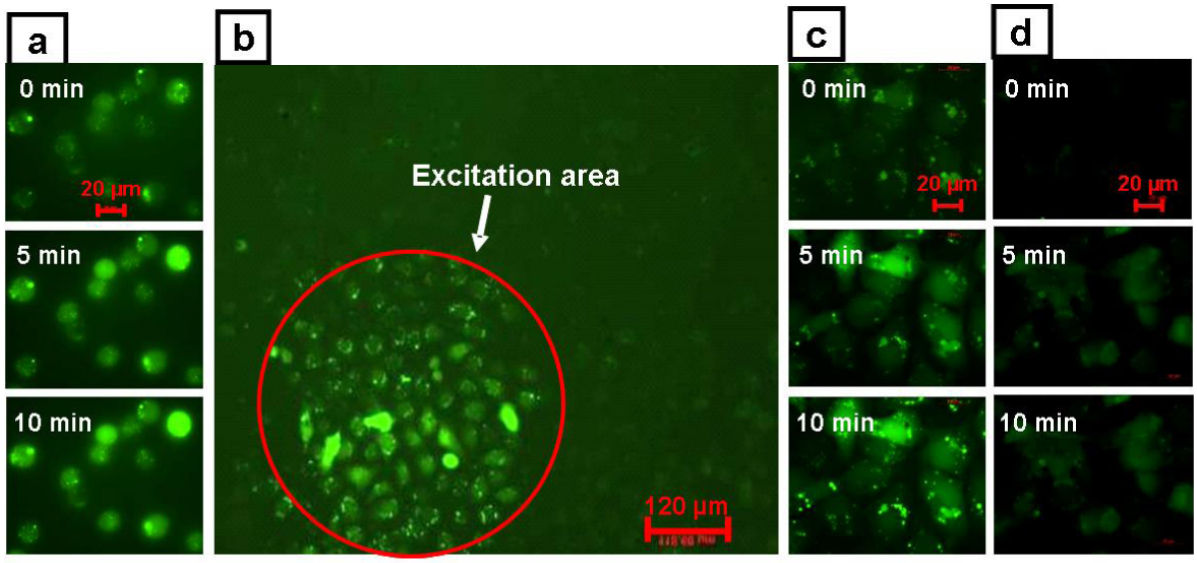

Figure 3. Fluorescence images of intracellular SWCNT–FC in SKOV-3 cells (a) after 2.5 s light exposure (480 nm), (b) with 10× objective after photo-activation of with 63× objective, (c) after PBS washing and 2.5 s light exposure, and (d) after PBS washing and 1.5 s light exposure.

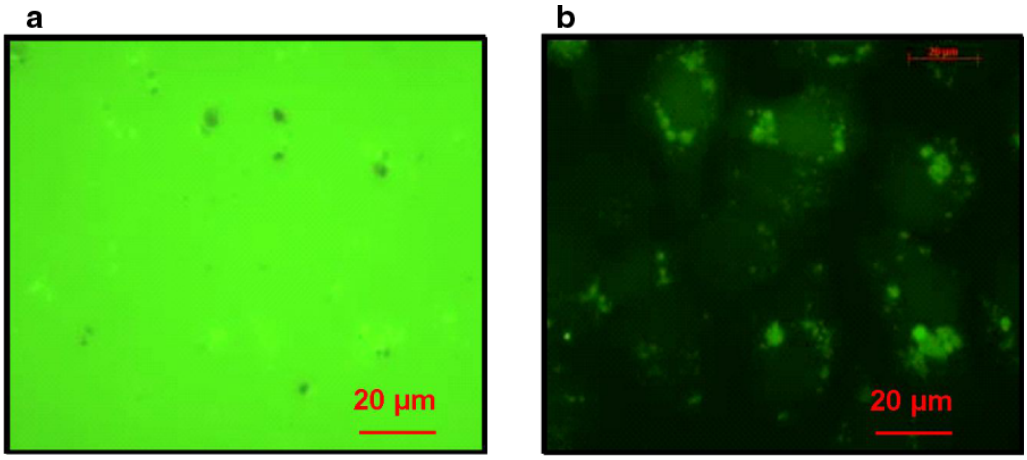

Figure 4. (a) Strong background fluorescence in the medium from SKOV-3 cells containing intracellular SWCNTs after 36 h incubation. (b) Same cells after washing with PBS.

respectively, after 10 min) (date were analyzed in figure 7). We found strong background fluorescence in growth medium after SWCNT–FC-loaded SKOV-3 cells (light-stimulated (480 nm)) were incubated for another 36 h (figure 4(a)), although the cells themselves retained their fluorescence after replacement of the medium (figure 4(b)). The presence of fluorescent material in the growth medium may be due to diffusion or expulsion of SWCNT–FC conjugates from the SKOV-3 cells to the external medium, or from endosomal hydrolysis of the conjugates that yields free and de-quenched FC molecules which are then expelled to the extracellular medium.

4. Discussion and conclusion

4.1. pH sensitivity of SWCNT–FC within cells

To explain the observed photo-activation of SWCNT–FC in SKOV-3 cells, we considered the pH sensitivity of FC fluorescence. FC photoluminescence increases with pH monotonically over the pH range 3–10 [23]. We confirmed this behavior for our SWCNT–FC conjugates by measuring the fluorescence intensity of aqueous solutions of SWCNT–FC conjugates at different pH values. Figure 5(a) shows that the fluorescence intensity of SWCNT–FC conjugates increases by two orders of magnitude from pH 4.1 to 7.0. An additional 20% increase occurs between pH 7.0 and 10.0. To investigate whether the intracellular photoluminescence of SWCNT–FC conjugates corresponds to changes in pH_i, we used chloroquine to modulate pH_i. Chloroquine is a weakly basic amine which concentrates in acidic intracellular compartments (lysosomes, Golgi complex) and increases the intracellular pH [24–28]. Figure 5(b) shows that the fluorescence intensity of intracellular SWCNT–FC conjugates increases after chloroquine is added to the medium (60% increase after 10 min) (date were analyzed in figure 7). Moreover,

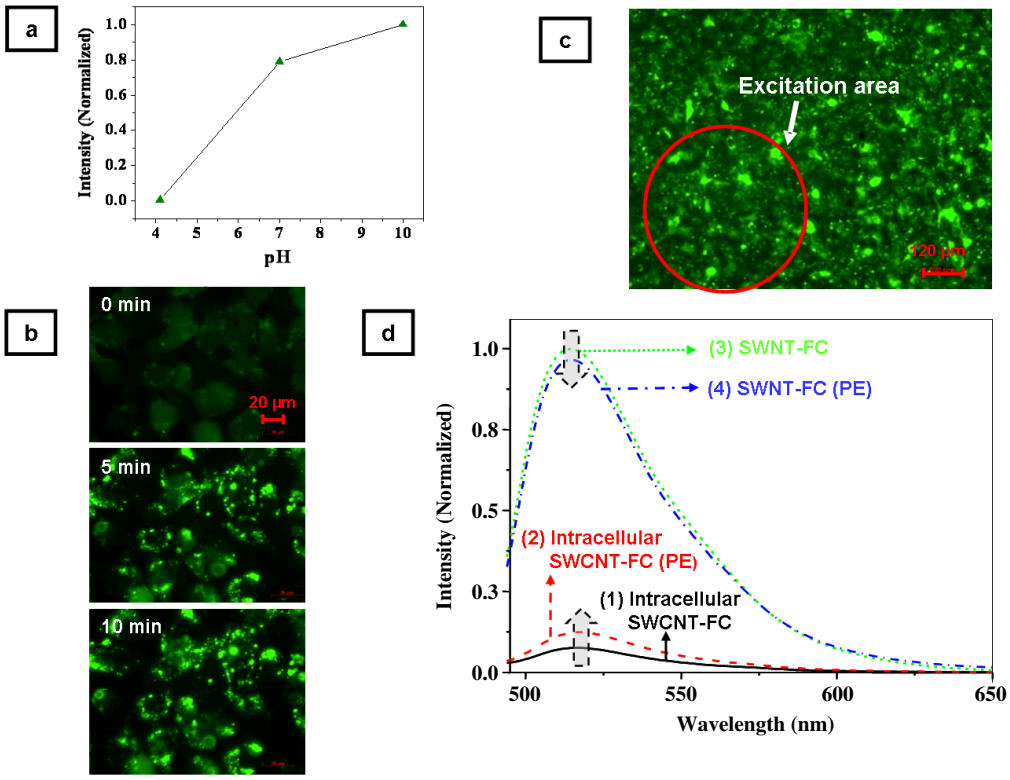

Figure 5. (a) Normalized fluorescence intensity of free SWCNT–FC conjugates increases with an increasing pH value within the range of 4.1–10.0. Fluorescence images of intracellular SWCNT–FC in (b) chloroquine-treated SKOV-3 cells after 2.5 s light exposure (480 nm), (c) with 10× objective after photo-activated with 63× objective. (d) Fluorescence emission intensity of free SWCNT–FC conjugates decreases after 480 nm exposure (photobleaching), while fluorescence emission intensity of intracellular SWCNT–FC conjugates increases after 480 nm light exposure.

chloroquine-treated, SWCNT–FC-loaded SKOV-3 cells photo-excited and imaged with 63× and 10× objectives display a uniform distribution of fluorescence emission intensity throughout the imaged sample (figure 5(c)), contrary to what is observed for photo-activated samples without chloroquine (figure 3(b)). Figure 5(d) shows fluorescence spectra of intracellular and extracellular SWCNT–FC conjugates before and after photo-excitation. Emission from extracellular SWCNT–FC conjugates is observed to decrease slightly (photobleaching) after excitation while photo-excited (480 nm) intracellular conjugates exhibit a strong fluorescence intensity increase. If SWCNT–FC internalization into SKOV-3 cells occurs via endocytosis, we may expect that the conjugates are localized in endosomal compartments, where they find a pH of around 6.0–6.5, before being delivered to lysosomes to be degraded. Lysosomes have an acidic internal pH of about 5.0 [29, 30]. In this environment the fluorescence of internalized SWCNT–FC conjugates will be substantially quenched. On the other hand, for internalized SWCNT–FC conjugates to increase their emission intensity, i.e. to be exposed to a higher pH environment, they must either cross the endosomal or lysosomal membrane into the cytoplasm, or the pH of the lysosomal compartment must increase. SWCNT–FC in the cytoplasm sees pH values around 7, which would correspond to a two- or four-fold increase in fluorescence intensity for endosomal and lysosomal conjugates, respectively [23]. Integration of the fluorescence intensities in figure 3 reveals a two-fold intensity increase for all photo-activated samples after 10 min, which matches closely the predicted intensity increase of SWCNT–FC conjugates due to endosome-to-cytoplasm transfer (date were analyzed in figure 7) or an increase in vesicle pH. Although it is possible for cationic conjugates to disrupt endosomal membranes [23], unreacted carboxyl groups in the SWCNT–FC conjugates would make them anionic at intracellular pH values.

4.2. Hydrolysis of SWCNT–FC within cells

An additional issue of concern in assessing the photo-activation process is the possibility of a photo-induced hydrolysis of the amide linkage between nanotubes and FC molecules. Fluorophores such as FC undergo strong fluorescence quenching when conjugated to carbon nanotubes due to energy transfer by π–π interaction between the nanotube and the dye molecules [31]. Therefore, hydrolysis of SWCNT–FC conjugates would in principle release de-quenched FC molecules, which in turn could also contribute to the photo-activated fluorescence intensity increase in SKOV-3 cells. Figure 5(d) shows fluorescence spectra of SWCNT–FC conjugates outside and inside SKOV-3 cells before and after photo-excitation. Emission from extracellular conjugates is observed to decrease slightly after photo-excitation while photo-excited intracellular conjugates underwent a strong

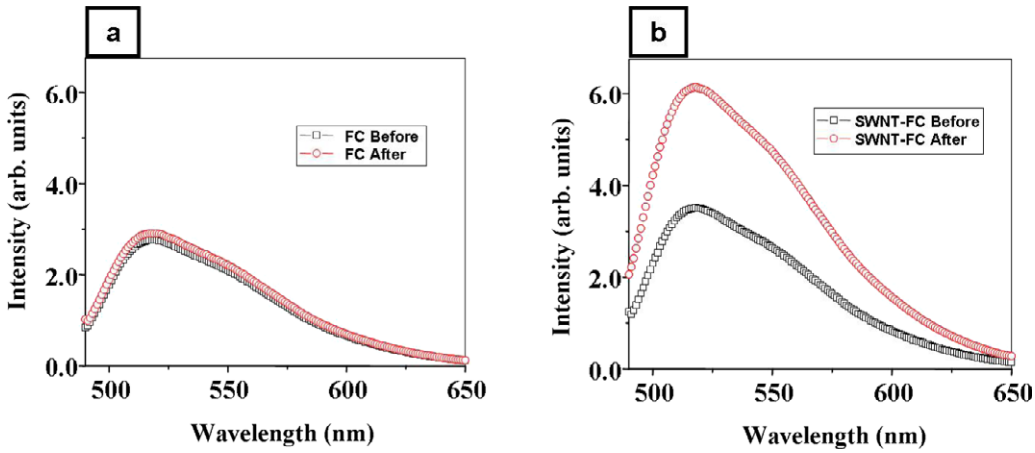

Figure 6. (a) Fluorescence emission of an FC solution remains unchanged after acid hydrolysis. (b) Fluorescence emission intensity of an SWNT–FC solution increases after hydrolysis.

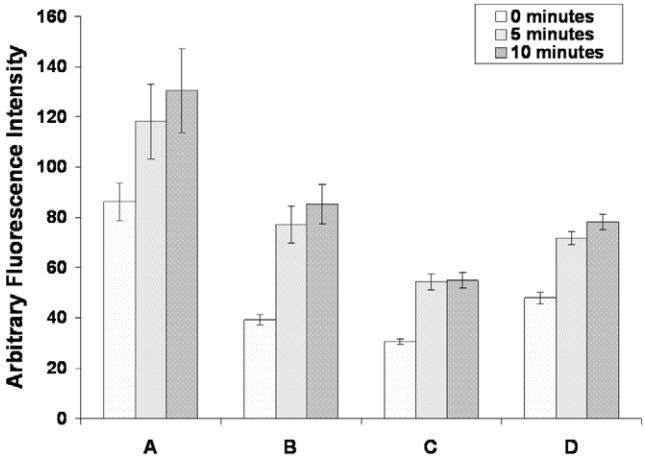

Figure 7. Intracellular fluorescence intensity from SWCNT–FC SKOV-3 cells was integrated and averaged with Zeiss AxioVision 3.1 imaging software. (a) After 2.5 s light exposure (488 nm), (b) after PBS washing and 2.5 s light exposure, (c) after PBS washing and 1.5 s light exposure, (d) after 2.5 s light exposure with chloroquine added (figure 5(b)).

fluorescence intensity increase. Hydrolyzable amide bonds that undergo photocleavage to give a free carboxyl group in significant yields are almost unknown [32]. Because the UV radiation required for amide photolysis falls in the range between 190 and 300 nm, therefore a pure photolysis of this bond during photo-activation (with light of 480 nm in wavelength) would not be favored. The fact that only intracellular conjugates yield a substantial fluorescence increase under light excitation clearly demonstrates the activation of one or several light-induced intracellular processes that may disrupt endosomal membranes and/or lead to the enzymatic hydrolysis of SWCNT–FC conjugates. Lysosomes carry hydrolases, which become active in the acidic intra-lysosomal environment and could cleave FC from nanotubes via hydrolysis. However, for large non-viral vectors, transfer from endosomes to lysosomes may be inhibited due to their size [33], therefore SWCNT–FC of comparable size may be hydrolyzed in endosomal compartments. This is consistent with the results shown in figure 3, for which the (two-fold) fluorescence increase matched better an endosome-to-cytoplasm rather than a lysosome-to-cytoplasm fluorophore transfer. To demonstrate that hydrolysis of SWCNT–FC conjugates could lead to fluorescence de-quenching we measured the fluorescence of free FC and SWCNT–FC conjugates before and after hydrolytic treatment. Briefly, free FC and SWCNT–FC conjugates were mixed with a 1:2 mixture of acetic and hydrochloric acids in a sealed tube at 110 °C for 48 h, for amide bond cleavage [34]. Figure 6(a) shows that the fluorescence intensity remains unchanged for FC solutions after the hydrolysis process. De-quenching due to hydrolysis of SWCNT–FC conjugates resulted in a nearly two-fold enhancement in fluorescence intensity (figure 6(b)), similar to the experimental data obtained with cells and consistent with the mechanisms proposed for the observations of internalized, photo-excited carbon nanotube conjugates in SKOV-3 cells.

The monotonic pH sensitivity of SWCNT–FC conjugates allows us to monitor the fate of SWCNT–FC conjugates in SKOV-3 cells. We find that SWCNT–FC conjugates are more likely taken up by an endocytotic process, for which the conjugates remain in endosomes. The observed photo-induced fluorescence increase of intracellular SWCNT–FC can be attributed to the activation of one or several processes that may include one or more of the following: hydrolysis of endosomal SWCNT–FC conjugates yielding free de-quenched FC molecules; permeation of the endosomal membrane to allow SWCNT–FC conjugates into the cytosol; neutralization of the acidic environment of the endosome. Although an unambiguous explanation of the photo-activation process remains to be determined, we have demonstrated the potential use of SWCNT–FC conjugates as localized intracellular probes.

Acknowledgment

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References

[1] Lin Y, Taylor S, Li H P, Fernando K A S, Qu L W, Wang W, Gu L R, Zhou B and Sun Y P 2004 *J. Mater. Chem.* **14** 527–41

[2] Sotiropoulou S and Chaniotakis N A 2003 *Anal. Bioanal. Chem.* **375** 103–5

[3] Pastorin G, Wu W, Wieckowski S, Briand J P, Kostarelos K, Prato M and Bianco A 2006 *Chem. Commun.* **11** 1182–4

[4] Zeni O, Palumbo R, Bernini R, Zeni L, Sarti M and Scarfi M R 2008 *Sensors* **8** 488–99

[5] Klumpp C, Kostarelos K, Prato M and Bianco A 2006 *Biochim. Biophys. Acta Biomembr.* **1758** 404–12

[6] Lin Y, Zhou B, Martin R B, Henbest K B, Harruff B A, Riggs J E, Guo Z X, Allard L F and Sun Y P 2005 *J. Phys. Chem.* B **109** 14779–82

[7] Heller D A, Baik S, Eurell T E and Strano M S 2005 *Adv. Mater.* **17** 2793–99

[8] Barone P W, Baik S, Heller D A and Strano M S 2005 *Nat. Mater.* **4** 86–16

[9] Heller D A, Jeng E S, Yeung T K, Martinez B M, Moll A E, Gastala J B and Strano M S 2006 *Science* **311** 508–11

[10] Graber M L, Dilillo D C, Friedman B L and Pastorizamunoz E 1986 *Anal. Biochem.* **156** 202–12

[11] Kashket E R 1985 *Annu. Rev. Microbiol.* **39** 219–42

[12] Rottenberg H 1989 *Methods Enzymol.* **172** 63–84

[13] Chacko V P and Weiss R G 1993 *Am. J. Physiol.* **264** C755–60

[14] Moon R B and Richards J H 1973 *J. Biol. Chem.* **248** 7276–8

[15] Schwiening C J 1999 *Regulation of Tissue pH in Plants and Animals a Reappraisal of Current Techniques* (Cambridge: Cambridge University Press)

[16] Al-Hilli S M, Al-Mofarji R T and Willander M 2006 *Appl. Phys. Lett.* **89** 173119

[17] Sun Y H, Liu Y S, Vernier P T, Liang C H, Chong S Y, Marcu L and Gundersen M A 2006 *Nanotechnology* **17** 4469–76

[18] Liu Y S, Sun Y H, Vernier P T, Liang C H, Chong S Y C and Gundersen M A 2007 *J. Phys. Chem.* C **111** 2872–8

[19] Chen J, Hamon M A, Hu H, Chen Y S, Rao A M, Eklund P C and Haddon R C 1998 *Science* **282** 95–8

[20] Hamon M A, Hu H, Bhowmik P, Niyogi S, Zhao B, Itkis M E and Haddon R C 2001 *Chem. Phys. Lett.* **347** 8–12

[21] Kam N W S, Liu Z A and Dai H J 2006 *Angew. Chem. Int. Edn* **45** 577–81

[22] Schwartz A L 1995 *Pediatr. Res.* **38** 835–43

[23] Ma L Y, Wang H Y, Xie H and Xu L X 2004 *Spectrochim. Acta* A **60** 1865–72

[24] Oda K and Ikehara Y 1985 *Eur. J. Biochem.* **152** 605–9

[25] Oda K, Koriyama Y, Yamada E and Ikehara Y 1986 *Biochem. J.* **240** 739–45

[26] Strous G J, Dumaine A, Zijderhandbleekemolen J E, Slot J W and Schwartz A L 1985 *J. Cell Biol.* **101** 531–39

[27] Maxfield F R 1982 *J. Cell Biol.* **95** 676–81

[28] Caplan M J, Stow J L, Newman A P, Madri J, Anderson H C, Farquhar M G, Palade G E and Jamieson J D 1987 *Nature* **329** 632–35

[29] Sipe D M and Murphy R F 1987 *Proc. Natl Acad. Sci. USA* **84** 7119–23

[30] Yamashiro D J and Maxfield F R 1987 *J. Cell Biol.* **105** 2713–21

[31] Wang F, Dukovic G, Knoesel E, Brus L E and Heinz T F 2004 *Phys. Rev.* B **70** 241403

[32] Amit B, Benefraim D A and Patchornik A 1976 *J. Chem. Soc., Perkin Trans.* 1 57–63

[33] Wattiaux R, Laurent N, Wattiaux-De Coninck S and Jadot M 2000 *Adv. Drug Deliv. Rev.* **41** 201–8

[34] Christensen H N and Hegsted D M 1945 *J. Biol. Chem.* **158** 593–600