Studies on the mechanism of action of a targeted chemotherapeutic drug in living cancer cells by two photon laser scanning microspectrofluorometry

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Abstract. In this study, we present a spectroscopic study of the entry pattern of a chemotherapeutic drug (AN-152) and its carrier hormone ([D-Lys⁶]LH-RH) into living cancer cells, with the help of our two-photon probes and a home-built localized microspectrofluorometer coupled with two photon laser scanning microscope (TPLSM). Due to the inherent localization ability of TPLSM, we were able to identify the drug and carrier location in different compartments of the cancer cells *in vitro*. The apparent doxorubicin-assisted nucleic accumulation of AN-152 suggests a possible nuclear action of the drug on cell proliferation. © *2001 Society of Photo-Optical Instrumentation Engineers.*
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1 Introduction

The imaging of drugs in living cells or deep within tissues of living animals is an important tool for studying the mechanism of the action of drugs and to monitor their actions in real time. Dynamic spectral profiles of a drug in living samples can provide quantitative or semiquantitative information about interaction with the intracellular environment. Recently, confocal laser scanning microscopy (CLSM) has been receiving more attention in pharmaceutical research as a three-dimensional imaging technique.[1] However, as most of the commercial probes require ultraviolet (UV) or visible excitation, CLSM imaging is often limited by photodamage and lower penetration depth due to the tissue's absorption and scattering in the UV and visible region.[2] In 1990, Denk et al. showed that two-photon laser scanning microscopy (TPLSM) with pulsed infrared excitation has an inherent localization ability with lower photodamage and better penetration into tissues.[3] Coupling TPLSM with a spectrometer, Masters et al. introduced the two-photon laser scanning microspectrofluorometer (TPLSMF) and recorded the spectra of NADH 80 μm under human skins *in vivo*.[4] Despite these advantages, TPLSM has rarely been used in pharmaceutical research. This is partially due to the lack of compatible probes with large two-photon excitation cross sections.

In this study, we present a spectroscopic study of the entry pattern of a chemotherapeutic drug, AN-152, and its carrier hormone luteinizing hormone release hormone peptide analog ([D-Lys⁶]LH-RH) into living cancer cells, with the aid of our two-photon probes and a home-built localized microspectrofluorometer coupled with TPLSM. Because of the inherent localization ability of TPLSM, we were able to identify the

location of the drug and carrier location in different compartments of the cancer cells *in vitro*.

AN-152 is a conjugate of the peptide analogy [D-Lys⁶]LH-RH and a chemotherapeutic agent doxorubicin (Dox). Dox has been used in the clinics for more than 30 yr, but cardiotoxicity and myelosuppression have greatly limited its efficacy.[5] AN-152 was designed to reduce the peripheral toxicity and improve the efficacy of Dox by targeting to LH-RH receptor-positive tumors such as breast, prostatic, ovarian, and endometrial cancers.[6–8] The advantages of the targeted chemotherapy based on LH-RH analogs have been well demonstrated during the past few years.[9] Our previous study on AN-152 by TPLSM showed that this drug can readily enter LH-RH receptor-positive MCF-7 breast cancer cells but not the LH-RH receptor-negative UCI-107 ovarian cancer cell line.[10] Its entry can even be regulated by activating and inactivating the LH-RH receptors on cells.[11] We also observed the apparent entry of a two-photon probe labeled AN-152 (AN-152:C625) into the nucleus, which implies a possible DNA intercalation of its cytotoxic moiety, Dox. However, occasional observation of the labeled [D-Lys⁶]LH-RH (LH625) in the nucleus of some cells raised questions about the role of the carrier (LH-RH analog) in the intracellular transport of the drug.

Monitoring of the intracellular distribution of AN-152 and the carrier LH-RH peptide analog simultaneously can provide a real-life comparison of the intracellular distribution of the drug and its carrier. Multichannel imaging by TPLSM can minimize the background from a light source, but it also requires different two-photon dyes with good spectral separations. Our previous success in optical tracking of the C625 labeled chemotherapeutic drug AN-152,[10,11] encouraged us to develop new probes with different emission ranges. We have

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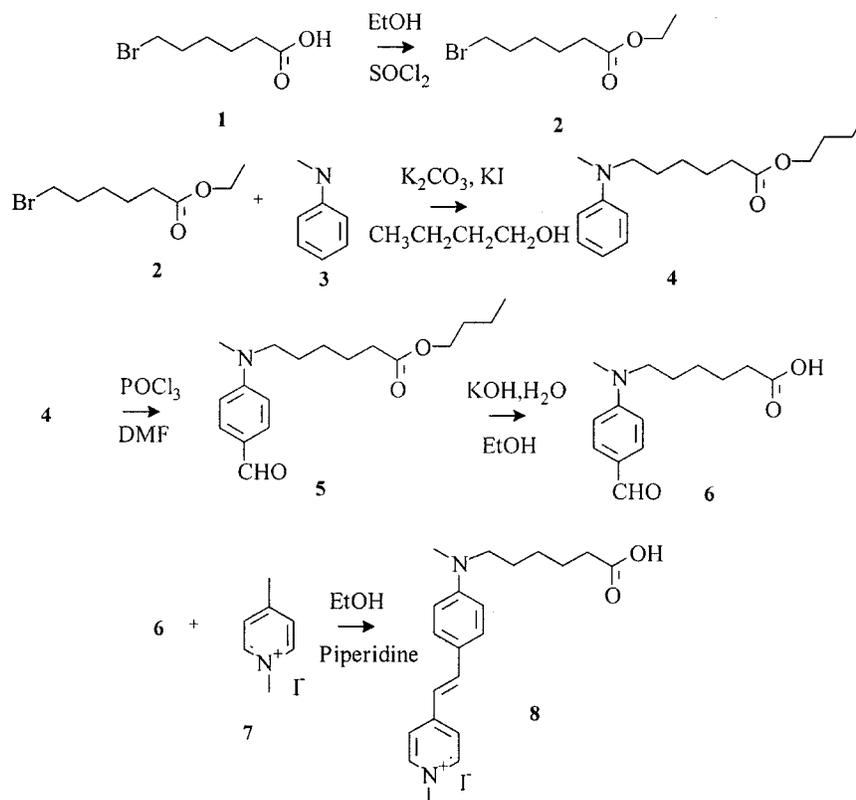
Fig. 1 Synthetic pathway of the two-photon probe TPR.

also modified our TPLSM setup to add the capability of acquiring two-photon excited localized fluorescence spectra inside the living cells. Using this TPLSMF setup and another two-photon probe in the red region (TPR), which emits in the red region, coupled to the peptide carrier [D-Lys^6]LH-RH, we were able to simultaneously study the spectra profiles of the drug and its carrier in a single living cell.

2 Materials and Methods

2.1 Two-Photon Laser Scanning Microspectrofluorometry (TPLSMF)

Two-photon confocal laser scanning microscopy images were obtained using a commercial CLSM, system model MRC-1024 (Bio-Rad, Richmond, CA), which was attached to an upright microscope (Nikon, model Eclipse E800). A water immersion objective lens (Nikon, Fluor-60X, NA=1.0) was used for cell imaging. The system was adapted for two-photon excitation at 820 nm by using dichroic mirrors and filters. A broad band-pass filter (460–600 nm) was used as an emission filter for imaging. The excitation light was provided by a Ti–sapphire laser (Spectra-Physics, Tsunami) pumped by a frequency-doubled diode pumped solid state laser (Spectra-Physics, Millennia). The system is operated at around 90 fs pulse width and 82 MHz repetition rate. The average power at the sample was adjusted to $\sim$ 15 mW to avoid photodamage to the cells.

For localized spectrofluorometry, the fluorescence signal was collected from the side port of the confocal microscope using a multimode optical fiber of core diameter 1 mm, and was delivered to a spectrometer (Holospec, Keiser Inc.)

equipped with a cooled charge coupled device (CCD) camera (Princeton Instruments) as a detector. The fluorescence signal was directed to the fiber by a dichroic mirror inserted into the sideport of the microscope, before passing through any filters inside the scanhead and without descanning. The system can be easily switched between TPLSM and TPLSMF by moving the dichroic mirror in and out of the beam path. The inherent optical sectioning capability of the two-photon process allowed us to do this collection without confocal aperture. An absorption filter in the infrared range was used inside the spectrograph to cut off the excitation line from the laser. The acquired spectra were corrected for the CCD response as well as for the transmission of the filters and mirrors in the spectral range of interest. Throughout the study, we normalized the obtained spectra with respect to the maximum intensity of the fluorescence spectra, as we were always using the ratio of fluorescence intensity peaks from two different fluorescent tags, rather than the individual intensity of one fluorescent tag.

2.2 Synthesis of Two-Photon Probe TPR and Conjugation of the Probes to AN-152 and [D-Lys^6]LH-RH

The chemotherapeutic drug AN-152 and the two-photon probe C625 were synthesized as described previously.[10,6] [D-Lys^6]LH-RH (Glp-His-Trp-Ser-Tyr-D-Lys-Leu-Arg-Pro-Gly-NH$_2$) was obtained from California Peptide Research, Inc. All other reagents were purchased from Aldrich and were used without further purification. The synthesis of the custom designed TPR is illustrated in Figure 1. The electron donor

part was synthesized by alkylation of N-methyl aniline with 6-bromocaproic acid ethyl ester. After the Vilsmeier reaction, the carboxylate protective group was removed by hydrolysis. The pyridinium derivative 7 was subsequently linked to amino-substituted aldehyde 6 through base catalyzed aldol condensation. The detailed strategies for the synthesis of this type of pyridinium salt have also been described in our previous work.[12] The final product, designated TPR, was characterized by proton nuclear magnetic resonance (NMR) and MS. [^{1}H NMR in CDCl$_3$ (500 MHz): δ1.29 (m, 2H), δ1.55 (m, 4H), δ2.17 (t, 2H), δ2.93 (s, 3H), δ3.34 (t, 2H), δ4.02 (s, 3H), δ6.70 (d, 2H), δ6.77 (d, H), δ7.40 (d, 2H), δ7.46 (d, 1H), δ7.66 (d, 2H), δ 8.21 (d, 2H); fast bombardment MS: calculated for $C_{21}H_{27}N_2O_2$ 339.2 found 339.2]. The preparation of the conjugate of C625 and AN-152 (AN-152:C625 or AC) and the conjugate of C625 and [D-Lys^6]LH-RH (LH-RH:C625 or LH625) was described in our previous work.[10] The conjugate of TPR and [D-Lys^6]LH-RH (LH-RH:TPR or LHTPR) was prepared with the same method approach. Purification of the labeled peptide products was carried out by Hewlett Packard Series 1050 HPLC equipped with a C$_{18}$ reverse phase analytical column (Phenomenex, Jupiter). All final products were characterized by matrix assisted laser desorption ionization mass spectroscopy.

2.3 Cell Culture

MCF-7, human breast cancer cells (American Type Culture Collection) were maintained at 37 °C in a humidified environment with 5% CO$_2$. Cells were cultured in improved minimum essential medium with 10% fetal bovine serum. Cells were subcultured in 30 mm petri dishes for 48–72 h (80% confluent). prior to incubation with labeled reagents.

2.3.1 *Two-Photon Localized Fluorescence Spectroscopy Inside Living Cells*

Fluorescent probes AC, LH625, LHTPR and TPR were used in our localized micro-spectro-fluorometric study in LH-RH receptor-positive MCF-7 cells.[10,11] The nucleus and the cytoplasm were identified by direct observation through transmission mode of the microscope, as well as by confocal reflection and fluorescence images. The specific areas inside the living cells were targeted, based on the two-photon excited fluorescence images acquired by TPLSM. The localized spectra with the resolution up to a diffraction-limited spot were then obtained by point or area scanning of TPLSMF.

2.3.2 *Ratiometric Study of the Localized Spectra in the Nucleus and the Cytoplasm*

All cells were pretreated with 10 nM epidermal growth factor (EGF) to upregulate the LH-RH receptors on the membranes.[11] As it is difficult to determine the fluorescence efficiency of dyes in different parts of a cell, we adopted a ratiometric approach in the analysis of the localized spectra from the labeled LH-RH analog and AN-152. Thus, regardless of the acquisition time (which did vary in the case of different environments), we calculated the ratio of the fluorescence peaks of C625 and TPR obtained from the spectra recorded at the same spot in the cell.
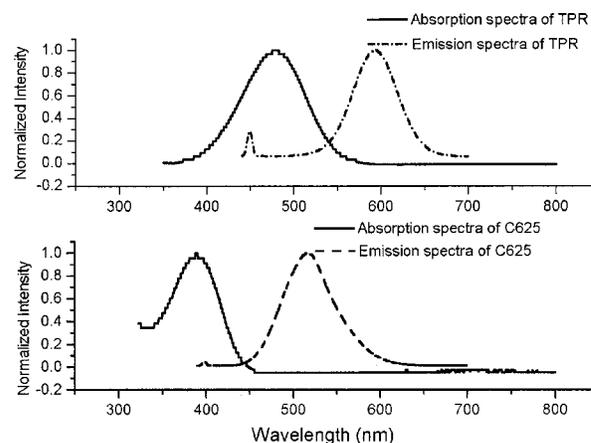

Fig. 2 Absorption and emission spectra of the probes TPR and C625 (in DMSO).

The ratio between the spectra of AC and LHTPR analog were compared inside the nucleus and cytoplasm of living cells. The control experiments include the localized spectra of the cells without any treatment, the cells incubated with C625 (dissolved in DMSO), and the cells treated with TPR itself. The acquisition time for localized spectra varied. The spectra from cytoplasm could be obtained in 30 s while it took up to 3 min to acquire the spectra from an apparently less stained nucleus.

EGF pretreated MCF-7 cells were treated with AC (0.6 μM) and LHTPR (3.8 μM). After 1.5 h, the localized spectra were taken outside the cells (background), and inside the cells (nucleus and cytoplasm). Similarly, the spectra from LH625 (0.6 μM) and LHTPR (3.8 μM) treated MCF-7 cells were taken as described above. In the control experiments, higher concentration of TPR (10 μM) was used with 0.6 μM AC.[10]

3 Results and Discussion

3.1 Results

A recently developed two-photon probe, C625, can be successfully used in real-time optical tracking using TPLSM.[10,11] The design of a new dye (TPR) with red color provides a nice probe with a different spectral window, even though it shows a lower two-photon fluorescence efficiency compared to C625. The absorption and emission spectra of TPR and C625 are shown in Figure 2. The excitation wavelength of 820 nm was selected to obtain reasonable excitation efficiency with both C625 and TPR. We used these probes to study the entry and transport of AN-152 and its carrier peptide in living cells *in vitro*.

In the control experiments, the cells treated with [D-Lys^6]LH-RH (but without addition of labeled molecules) were scanned for 1 h to check for autofluorescence and no significant emission was detected. To observe the entry ability of TPR itself, we directly added TPR (10 μM) and AC (0.6 μM) to the medium of cells. As AC can enter MCF-7 cells and even stain the nucleus within 1 h,[11,10] its high fluorescence efficiency allows for an easier identification of the nuclei inside the cells from fluorescence images. Figure 3(a) shows the localized spectra inside the nucleus, cytoplasm, and outside the cells, 90 min after staining. The white and black
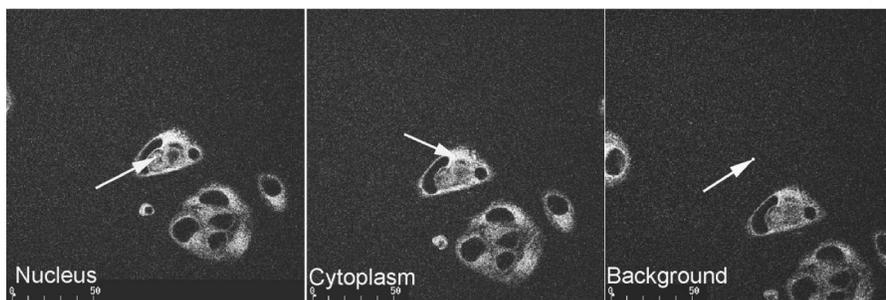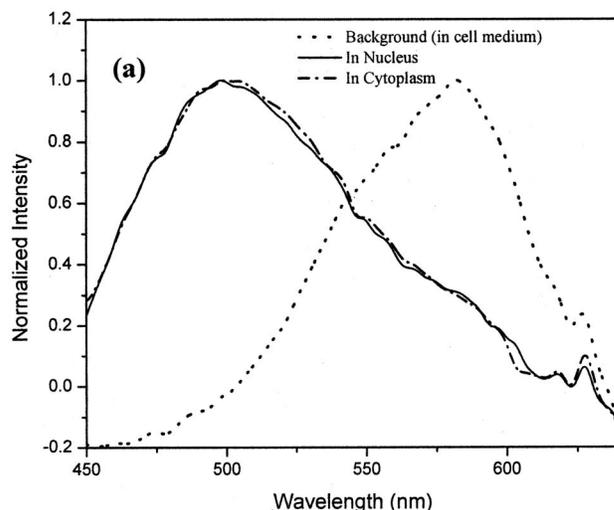
Fig. 3 (a) Localized spectra from the nucleus, cytoplasm, and medium from an AC&TPR treated MCF-7 breast cancer cell. TPR (10 μM) and AC (0.6 μM) were added to the medium of cells and localized spectra were acquired 90 min later. All the acquired spectra were normalized with respect to the maximum fluorescence intensity. (b) Two-photon confocal microscope images showing the positions where the spectra were acquired.

spots in the center of each image in Figure 3(b) correspond approximately to the size of the focus point when the spectra were being taken. From Figure 3, it can be seen that the localized spectra inside the cells (nucleus and cytoplasm) are dramatically different from the one obtained outside the cell.

To investigate the effect of the local environment on the fluorescence efficiency of AC and TPR, we recorded their fluorescence spectra in water and after adding membrane model dimyristoyl-L-α-phosphatidylcholine (DMPC). Figure 4(a) shows the emission spectra of AC in water before and after adding DMPC. The dramatic improvement of fluorescence from AC after it is associated with the model membrane can clearly be seen. In contrast, the emission of TPR changed little after adding DMPC to the solution [Figure 4(b)].

To study the effects of intracellular environment on the fluorescence efficiency of the probes C625 and TPR, we coupled them to [D-Lys^6]LH-RH to form LH625 and LHTPR, respectively. LH625 and LHTPR were then added to the medium of MCF7 cells and the fluorescence spectra from the nucleus, the cytoplasm, and the media recorded (Figure 5). The peaks from LH625 and LHTPR in the medium are readily observed. The calculated ratiometric spectral profiles of LHTPR and LH625 were similar in the nucleus and the cytoplasm.

In other experiments, we added AC and LHTPR to MCF-7 cells and studied the spectroscopic profiles inside the cells. Figure 6(a) shows the localized spectra from the nucleus, the cytoplasm of an MCF-7 cell. The cell was pretreated with

EGF to upregulate the number of LH-RH receptors on the membrane of cells,[11] and then treated with AC (0.6 μM) and LHTPR (3.8 μM). LHTPR concentration used in this case is considerably lower than that of TPR (10 μM) in the control experiment. Figure 6(b) shows the spectrum taken from the background without any cells, where we can clearly see that the emission from LHTPR is dominating.

This experiment was repeated in different cultures of MCF-7 cells and the spectra were taken in several cells of each culture. The spectroscopic profiles of the nucleus and the cytoplasm were reproducible. Occasionally, the fluorescence intensity of AC and LHTPR in the cytoplasm was found to be similar, but in all cases the ratio of AC to LHTPR in the nucleus (2.46 ± 0.53) was significantly higher than that in the cytoplasm (0.88 ± 0.45) (as calculated from the peak fluorescence intensities of the fluorescent tags C625 and TPR, respectively).

In another experiment, we intentionally induced photodamage to the cell by increasing the laser power. The dynamic change of the spectral profile in the nucleus can be clearly observed by TPLSMF [Figure 7(a)]. Figure 7(b) shows the fluorescence images of the same cell before and after photodamage, respectively, which could be easily identified through the observation of morphology changes by transmission and fluorescence microscopy. Our procedure was to first acquire the localized spectra (Δ) from the nucleus of the cell, and then induce photodamage to the cell by increasing the power of the
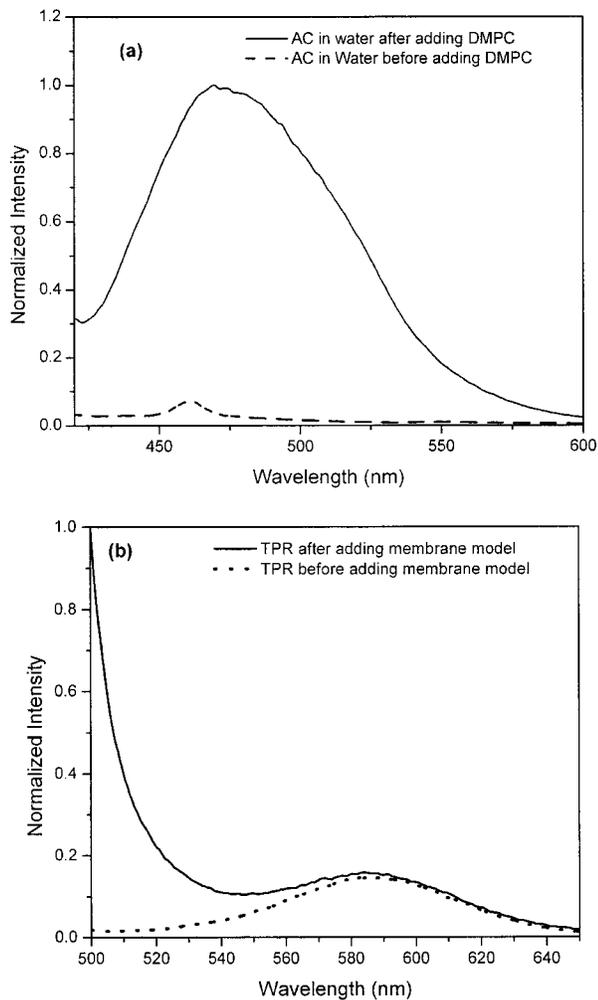
Fig. 4 Emission spectra of AC (a) and LHTPR (b) in water and while bound to lipid vesicles [dimyristoyl-L-α-phosphatidylcholine (DMPC)]. AC shows much stronger fluorescence when it is associated with membranes (more hydrophobic environment) while TPR shows almost same fluorescence efficiency with and without model membranes.
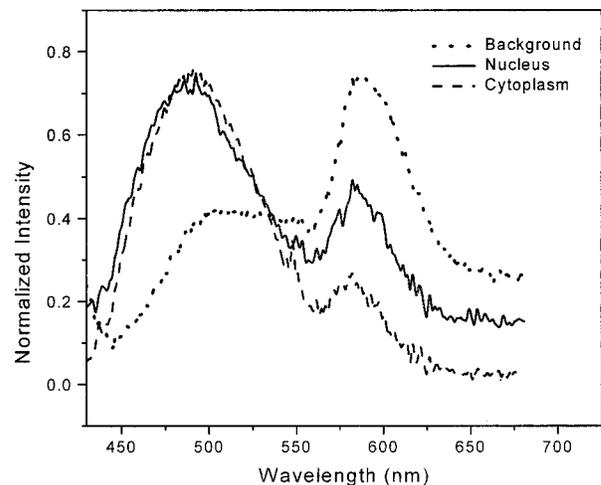

Fig. 5 Localized spectra from the nucleus and cytoplasm of an LH625 and LHTPR treated MCF-7 breast cancer cell and its background spectra from the media. LH625 (0.6 μM) and LHTPR (3.8 μM) were added to the medium of cells and localized spectra were acquired 90 min later. All the acquired spectra were normalized with respect to the maximum fluorescence intensity.

laser. At that time, another spectra ($\bigcirc$) was acquired from the nucleus by shifting the focus a small distance away to avoid the photobleached region. A dramatic difference in profiles before and after cell damage was observed. While the emission intensity from AC was much higher than that of LHTPR in the nucleus of the living cell, the emission from LHTPR virtually dominated in the case of the photodamaged cell.

We have also used a single-photon confocal microscopy to image the entry of unlabeled Dox into the MCF-7 cells.[11] Dox shows some fluorescence with an excitation wavelength of 488 nm. Its entry into cells was monitored by one-photon confocal microscopy using the same equipment as described above, but equipped with an emission filter for 580 nm (bandpass filter 580/32). Figure 8 shows a single photon image of an MCF-7 cell treated with Dox (1 μM).

3.2 Discussion

In MCF-7 cells treated with TPR (10 μM) and AC, the spectra of the nucleus and the cytoplasm are very similar and the peak

positions are close to the emission spectrum of C625 in chloroform (Figure 3). There is no spectroscopic indication of the presence of TPR inside the cells or at least, the entry of TPR into the cells was so negligible that their spectrum was overridden by the emission of AC, even though the concentration of TPR (10 μM) used in this experiment was much higher than the concentration of LHTPR (3.8 μM) used in other experiments. In contrast, the spectra obtained from the medium showed no emission peak from AC, while we could clearly see the peak corresponding to the emission of TPR. The lack of signals from C625 in the medium can be due to the low fluorescence efficiency of AC in water, and also to a high degree of internalization. The strong emission signals for AC inside the cells could be a result of the dramatic increase in its fluorescence efficiency during the interaction of the lipophilic C625 moiety with cellular membranes as demonstrated with the model membrane, DMPC (Figure 4). The lipophilic property of the C625 moiety of AC actually aids in understanding the intracellular environment of the drug.

In the case of cells treated with AC and LHTPR, although the spectra obtained from the media and the cells differed, all the spectra taken inside the cells, including the nucleus, showed the emissions from both AC and LHTPR. These are dramatically different from the spectra in Figure 3, in which TPR (10 μM) instead of LHTPR (3.8 μM) was used together with AC. The spectroscopic exclusion of TPR from MCF-7 cells made it an excellent probe to track the entry of LHTPR. This result supports our previous observation that LH-RH could also enter the nuclei of LH-RH receptor positive cells.[10,11] Recently the presence of nuclear binding sites for LH-RH in the nuclear fraction of the LH-RH receptor-positive OV-1063 human ovarian cancers was also demonstrated by radioreceptor assays.[13] On the other hand, we found different intensity ratio profiles between AC and LHTPR emission in different compartments of cells. The spectra in Figure 6 correspond to the emission from the points shown in Figure 4(b), which were identified by us with transmission and fluores-
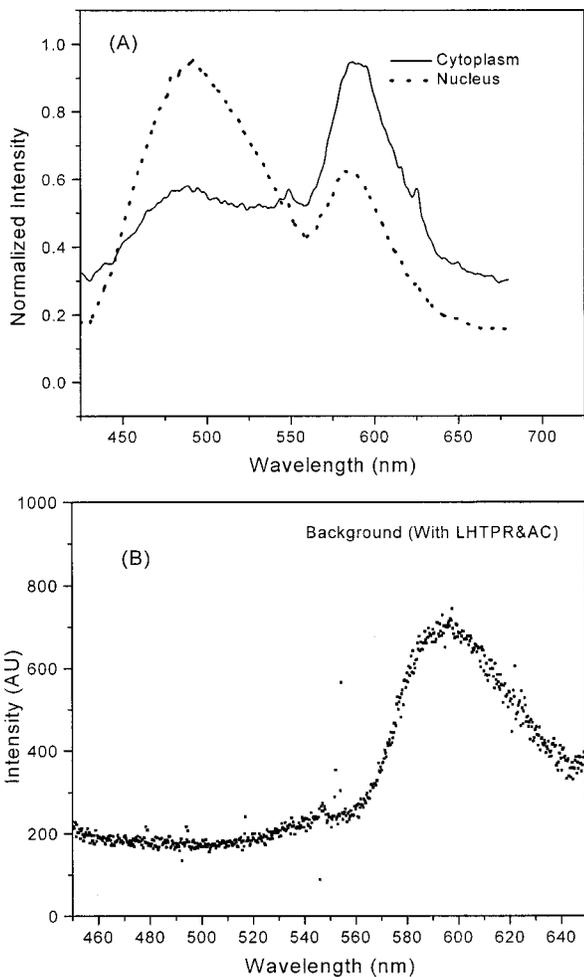
Fig. 6 Localized spectra from the nucleus and cytoplasm of an AC and LHTPR treated MCF-7 breast cancer cell and its background spectra from the media. AC (0.6 μM) and LHTPR (3.8 μM) were added to the medium of cells and 90 min later, localized spectra were acquired from different compartments of the cell and from the medium. All the acquired spectra were normalized with respect to the maximum fluorescence intensity.

cence microscopy as the nucleus or cytoplasm. Our results indicate that the ratio of the peak fluorescence values between AC and LHTPR inside the nucleus is consistently greater than in any other part of the cell.

This ratiometric difference cannot be due to the changes in fluorescence efficiency of the two probes, in different cellular environment, as the cells showed similar ratiometric spectral profiles after the treatment with LHTPR and LH625 (Figure 5). The study on the intracellular spectral patterns of LHC625 and LHTPR also ruled out another possibility that probes may cause the distribution difference of AC and LHTPR in MCF-7 cells.

In contrast to living cells, the fluorescence from LHTPR in the nucleus of dead (photodamaged) MCF-7 cells was much higher than that from AC, as shown in Figure 7. This dynamic change may be due to the "leaky" membranes of the damaged cell. After the photodamage occurs, the nucleic membranes become more permeable and less selective to the molecules outside. Because there are no membrane structures inside the
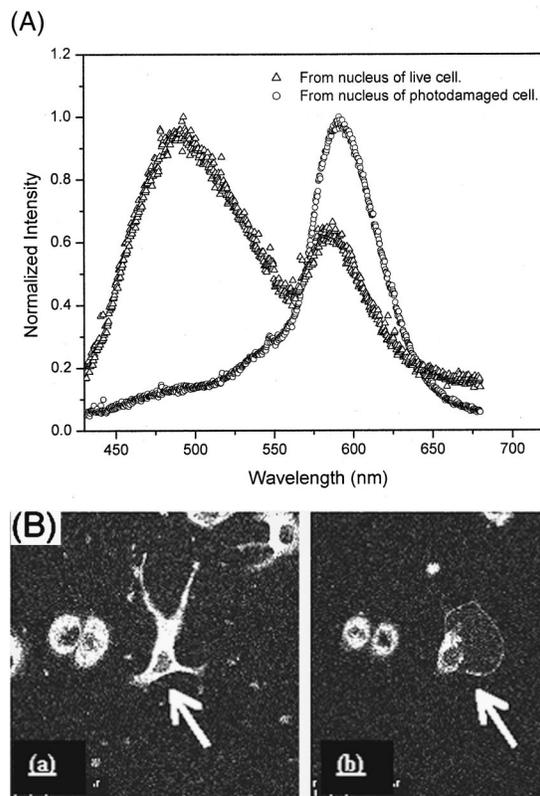

Fig. 7 (a) Localized spectra from the nucleus of an AC and LHTPR treated MCF-7 breast cancer cell before and after photodamage. After acquiring the localized spectra(Δ) from the nucleus of the cell, we intentionally induced photodamage to the cell by increasing the power of the laser. The other spectra ($\bigcirc$) were acquired from the nucleus by shifting the focus a small distance to avoid the photobleached region. (b) Fluorescence images of live cell (a) and dead cell (b). The white arrows in the picture indicate the cell from which the spectra were recorded.

nucleus like those in cytoplasm and AC has much weaker fluorescence in a hydrophilic environment, the nucleic fluorescence from LHTPR would become dominant. Conversely, when the cells are healthy, it could be much more difficult for LHTPR than for AC to enter the nucleus.
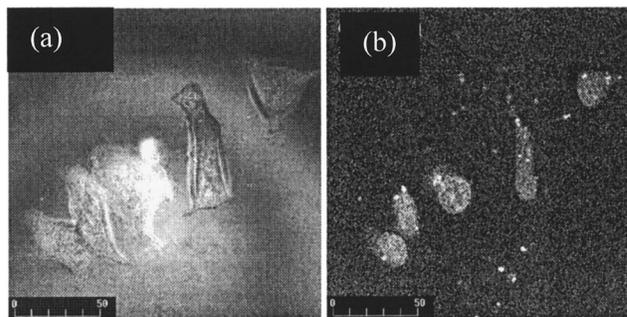

Fig. 8 Single photon confocal microscopy imaging of Doxorubicin in MCF-7 breast cancer cells using a 488 nm line from a Kr:Ar laser as an excitation source. A 580/30 emission filter was used in front of the fluorescence detector. The cells were incubated with Doxorubicin for 30 min before imaging: (a) reflection mode image and (b) fluorescence mode image.

As we do not know the fluorescence efficiency of C625 and TPR inside the cells, we cannot quantitatively correlate their intensities with the local concentrations. However, at the same local environment, we can still characterize their dynamic changes in relative concentrations from the ratio of their spectral intensities. The differences in the emission profiles in the nucleus and the cytoplasm imply a different ability of AN-152 and [D-Lys^6]LH-RH to enter the nucleus. AN-152 is a cytotoxic conjugate of Dox and [D-Lys^6]LH-RH. As one of the most widely used anticancer drugs, Dox has been shown to have a broad spectrum of antitumor effect.[14] Even though Dox has been used in the clinics for about 30 yr, its mechanism of action is still not completely clear and has been the subject of intense investigations.[5] Present concepts about the toxic effect of Dox are that it could initiate DNA damage through inhibition of topoisomerase II, after intercalation into the DNA, which could happen only inside the nucleus. The mechanism of action of the hormone conjugate AN-152 should also be elucidated. Our previous research has shown its cellular pathway into the nuclei of LH-RH receptor-positive cells, but it is not certain whether this entry was caused by the Dox component or the peptide. From the localized spectroscopic profiles obtained by TPLSMF, it appears that AN-152 can accumulate in the nucleus more readily than the carrier itself.

Furthermore, our previous study[11] using single photon confocal microscopy, has shown that Dox without any carrier can accumulate in the nuclei of MCF-7 cells. From Figure 8 in this study, it is clear that Dox is present in the nucleus at much higher concentrations than in the cytoplasm of MCF-7 cells. Therefore, it is reasonable to assume that the Dox part of AC is responsible for the higher relative concentration of AC in the nucleus. This study provides the first visual evidence that AN-152 may have a cytotoxic mechanism of action similar to that of Dox.

4 Conclusions

In this study, a spectrometer was successfully coupled with TPLSM to build a two-photon laser scanning microspectrofluorimeter system. Two-color tagging using two-photon fluorophores, optical tracking, and localized spectroscopy were used to investigate simultaneously the entry pattern of the targeted chemotherapeutic drug (AN152) and its peptide carrier into the cytoplasms and nuclei of living MCF-7 breast cancer cells *in vitro*. The combination of two-photon imaging, the ratiometric study of the spectra profiles, and the spectral shift reveals more information about the mechanism of action of AN152 in living cells. This work also proves the potential of TPLSMF in the study of the dynamics of drugs in living samples. The inherent advantages of two-photon excitation may make TPLSM and TPLSMF promising tools in the study of highly scattering samples like thick tissues and even living animals.

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