Study of fluorescence of doxorubicin in muscle tissue using highly sensitive fluorescence sensing

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Received 12 Mar 2013; Accepted 16 Apr 2013; Available Online 16 Apr 2013

Abstract

Monitoring the behaviour of biologically active compounds in the body is the key to understand their effect. Optical methods are relatively cheap non-ionizing techniques based on the specific optical properties and are an important tool for non-invasive and objective diagnosis with still better and better resolution. In this work, a characterization of fluorescence behaviour of doxorubicin in different environments (ethanol, methanol, dimethyl sulphoxide) has been established. In addition, its spatial distribution in muscle tissue was monitored using fluorescence imaging. Autofluorescence of the tissue was removed by software to limit the effect of the matrix. The novelty of this study lies in the field of sensing of doxorubicin in a tissue. The results can be base for an optic array, which could be used for sensing if the drug in vivo.

Keywords: Doxorubicin; Fluorescence; Nanomedicine; Fluorescence spectroscopy; *In vivo* imaging; Nanotechnology

1. Introduction

Malignant diseases represent 25% of the cause of death in developed countries. Colorectal cancer, breast cancer, prostate cancer and lung cancer are the most common types of cancer. On the other hand, mortality caused by these diseases decreases and survival time increases due to early diagnosis and effective treatment [1]. Magnetic resonance imaging (MRI), ultrasound (US), positron emission tomography (PET), computed tomography (CT), single-photon emission computed tomography (SPECT) and optical imaging methods belong to the contemporary used imaging methods in diagnostics and monitoring of the therapy [2,3]. Optical methods are relatively low cost non-ionizing methods based on the specific optical properties. They represent an important tool for non-invasive diagnosis with still developing properties as resolution [4,5]. It is advantageous to use inherent fluorescent properties of substances and labelled molecules [6]. Fluorescence reflectance imaging (FRI) enables imaging of fluorescent probes in tissues. In this case, the source of radiation and detector are at the same side of the object. Connection of laser and sensitive CCD device together with advanced mathematical models allows sensitive detection and evaluation of fluorescence intensity. Fluorescence-mediated molecular tomography enables reconstruction of three dimensional images of fluorescent probes in a tissue [7-9]. Organic fluorophores (fluorescein, rhodamine), biological fluorophores

(green fluorescence protein), or quantum dots can be sources of fluorescence for these purposes. In addition, inherent fluorescence of some drugs (doxorubicin, ellipticine) can be used [6,10-12]. In the area of basic research, detection of fluorescence of therapeutics is beneficial particularly in the development of targeted therapy and control of drugs targeting into place affected by a tumour. Doxorubicin is a highly effective and widely used anthracycline antibiotic, important antineoplastic drug intercalating DNA and causing oxidation stress that is used to treat leukaemia and solid tumours [13-19]. However, its application is limited by high cardiotoxicity, therefore it is necessary to monitor the applied dose [11,17].

Stationary techniques, such as spectrophotometric methods [20] including fluorimetry [21-23], but also separation methods, such as high performance liquid chromatography [24-28] and capillary electrophoresis [29-32], can be used for *in vitro* characterisation and pharmacological evaluation of doxorubicin. *In vivo* studying of interactions and distribution of doxorubicin can be performed in a microscale using microscopic techniques in different arrangements as laser scanning microscopy [33-35], fluorescence life time microscopy [14] and scanning electron microscopy [36]. At the macroscale level, high-frequency ultrasound imaging [37], PET imaging [38] and fluorescence imaging using quantum dots [39] have been used for the monitoring doxorubicin or doxorubicin-modified nanoparticles in tissues.

The aim of this work was to study the fluorescent properties of doxorubicin and to study the behaviour of doxorubicin diluted to different concentrations in water or methanol. In addition, doxorubicin was injected into muscle tissue to monitor its behaviour and to detect its fluorescence (emission) at different depths because of the fact that a major problem with the clinical use of doxorubicin in addition to adverse side effects common to all cytostatics like myelosupression, nausea and vomiting, mouth ulcers, local agressivity and alopecia, is their cardiotoxicity. Cardiotoxicity limits administration of doxorubicin exceeding an accumulated dose of approximately 450-550 mg/m^2 [40]. The toxic effects of anthracyclines to cardiomyocytes are not the result of inhibition of DNA synthesis, because these cells do not replicate [41]. The mechanisms of the anthracyclines cardiotoxicity are not fully understood, but cardiac tissue is vulnerable to free radical because of the low activity of antioxidant enzyme systems in cardiomyocytes altered by the effect of doxorubicin [17]. From the point of view of cardiotoxicity, doxorubicin and endogenous formaldehyde form a conjugate of two anthracycline molecules with three methylene groups, two forming oxazolidine rings and one binding the oxazolidines together at their 3´-amino nitrogens. This conjugate may hydrolyze to produce an active monomeric metabolite in which the carbon of formaldehyde is recovered in the form of a Schiff's base at the aminogroup of daunosamine [41, 42]. Anthracycline-formaldehyde conjugates intercalates into DNA by covalent bonding of the Schiff's base with the 2-amino group of a G-base in the minor groove of DNA. If the interaction with DNA occurs at the trinucleotide 5´-NGC-3´, then the drug intercalates between N and G and covalently bonds to the G-base on one strand using formaldehyde, and to the G-base on the opposing strand using hydrogen bonds. This combination of intercalation, covalent bonding, and hydrogen bonding is referred to as the *virtual cross-linking* of DNA by anthracyclines [43].

2. Experimental Details

2.1. Reagents and solutions

The doxorubicin infusion solution (Doxorubicin-Teva 0.2 %) was purchased from Teva Pharmaceuticals (Czech Republic). The solution contains doxorubicin (2 mg/ml), sodium chloride, hydrochloric acid (1 mol/l, used for pH adjustment), sodium hydroxide (for pH adjustment) and water.

2.2. Fluorimetric analyses

The doxorubicin solutions were prepared by gradually diluting (4 – 2000 µg/ml) in water or in methanol. The fluorescence spectrum was measured and the parameters were as follows: excitation wavelength: 480 nm, emission wavelength range: 520 – 850 nm; gain: 50; number of flashes: 5; emission wavelength step size: 5 nm. Fluorescence spectrometer Tecan infinite M200 PRO (Grödig, Austria) was used for these measurements. A volume of 50 µl of the solution was measured in the Nunc microtitration plates MaxiSorp (Thermo Fischer Scientific, Waltham, USA).

2.3. Highly sensitive CCD detector

The fluorescence properties were tested by Carestream In-Vivo Xtreme Imaging System (Carestream Health, Inc., Rochester, USA), parameters were set as follows: excitation wavelength - 480 nm, emission wavelength -

600 nm, exposure time - 1 second, binning - 1x1, *f*-stop - 1.1, field of view - 15 x 15 cm. The 4MP Camera is a cooled back-thinned, back illuminated camera designed for maximum sensitivity. The camera utilizes a two-stage thermo-electric cooler that cools down the CCD below -55 °C absolute. The camera collects the image data on a 2048 x 2048 pixel CCD. Single frame image data is digitized at 16-bits, and presented in software as a 32-bit floating point image. The images were processed by Carestream molecular imaging software (Carestream).

2.4. Application of doxorubicin in the muscle tissue

The doxorubicin was applied by injection into the muscle tissue (chicken breast) by insulin injection (Chirana T. injecta, volume: 1 ml, 0.33 x 12 mm). In addition, we used also an infusion with peristaltic pump (Pump P-1, Amesham Biosciences, AP Czech) with flow rate set to 100 µl/min. Doxorubicin was also applied into the tube (internal diameter of 1 mm) and it was inserted into the different depths of the tissue. The images were analysed by Carestream molecular imaging software (Carestream Health, Inc., Rochester). The intensity of fluorescence and the area of the emission of doxorubicin in the tissue were measured. To eliminate the autofluorescence effect, the spectral unmixing fluorescence software (Carestream Multispectral) enabling to distinguish between the signal of the tissue and the fluorophore was used. We test different excitation wavelengths (410 – 550 nm) and different tissue depths of application/insertion (0, 1, 2, 3, 4, 5, 6, 7 mm).

2.5. Descriptive statistics

Data were processed using MICROSOFT EXCEL® (USA) and STATISTICA.CZ Version 8.0 (Czech Republic). Results are expressed as mean ± standard deviation (S.D.) unless noted otherwise.

3. Results and Discussion

3.1. Fluorescence properties of doxorubicin

Fluorescence properties of substances are generally given by their structure and the surrounding environment. Absorption (excitation) and emission maximum of Doxorubicin is 480 nm and 600 nm, respectively. These properties are determined by the system of conjugated bonds in its structure (Figure 1). In this study, the behaviour of doxorubicin in different environments was studied. It has been established that the organic solvents (methanol, ethanol, dimethyl sulphoxide (DMSO), glycerol) significantly increase the fluorescence (intensity of emission) of doxorubicin (data not shown). It was proved that the solvents such as ethanol and methanol have the greatest effect on the intensity of emission of doxorubicin. The intensity of fluorescence of doxorubicin dissolved in methanol was almost 5 times higher than the intensity of fluorescence of doxorubicin dissolved in water (Figures 2A and B). As expected, doxorubicin fluorescence increased with its increasing concentration. The highest intensity was determined for doxorubicin at the concentrations of 63 – 125 µg/ml (Figure 2C). For doxorubicin diluted in methanol it was in the concentration range from 125 to 500 µg/ml (Figure 2D). Application of higher concentrations of doxorubicin led to a large concentration quenching and therefore lower fluorescence signal [44].
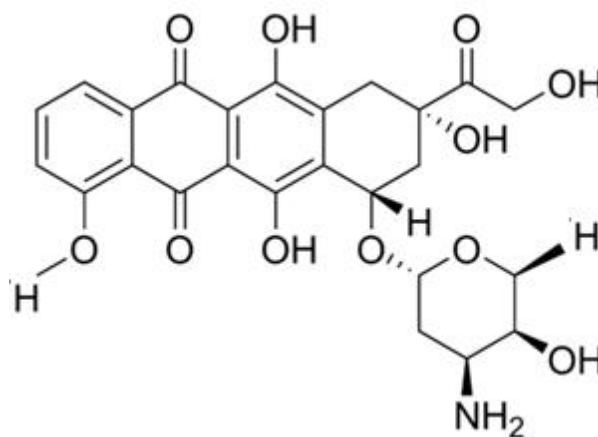
Figure 1. Chemical structure of doxorubicin based on the system of conjugated bonds.
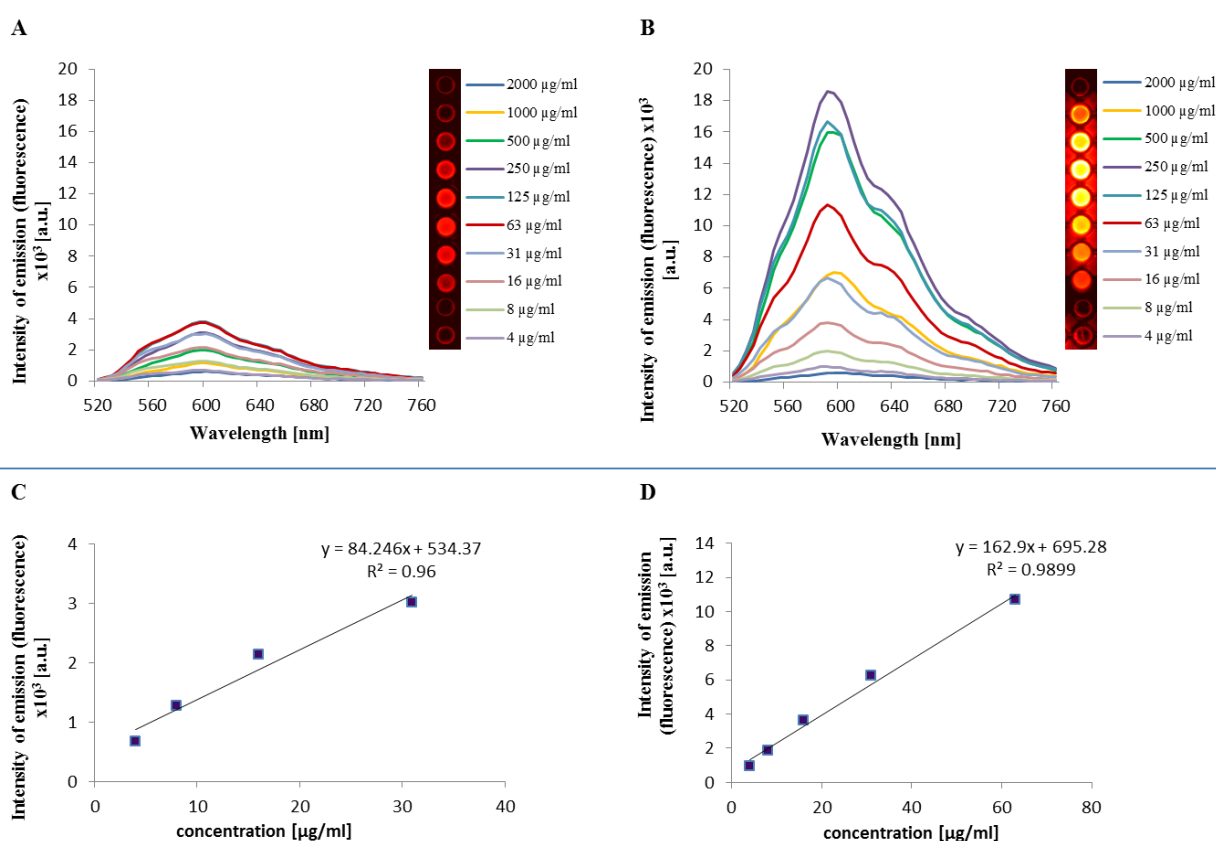

Figure 2. The dependence of the intensity of fluorescence on the concentration of doxorubicin: (A) the fluorescence spectra of the different concentrations of doxorubicin dissolved in water; (B) the fluorescence spectra of the different concentrations of doxorubicin dissolved in methanol; (C) the dependence of the intensity of fluorescence on the concentration of doxorubicin dissolved in water; (D) the dependence of the intensity of fluorescence on the concentration of doxorubicin dissolved in methanol (excitation wavelength: 480 nm, emission wavelengths: 520 - 760 nm).

The linear dependence of fluorescence intensity on the concentration of the doxorubicin was observed up to the doxorubicin concentration of 31 μg/ml dissolved in water or 63 μg/ml of doxorubicin dissolved in methanol (Figure 2).

3.2. Analysis of doxorubicin by highly sensitive CCD detector

The fluorescent properties of doxorubicin were also studied using highly sensitive fluorescence CCD detector (excitation: 480 nm, emission: 600 nm). Results obtained by *in vivo* fluorescence imaging system were comparable to results obtained using the fluorescence spectrometry. However, fluorimeter is a better tool for the quantification of fluorescence intensity due to the higher intensity of the excitation radiation since the intensity of detected fluorescence radiation (emission) is directly proportional to the excitation radiation intensity [45]. Nevertheless, *in vivo* fluorescence detector allows the fluorescence detection in a three dimensional area with a relatively high resolution.
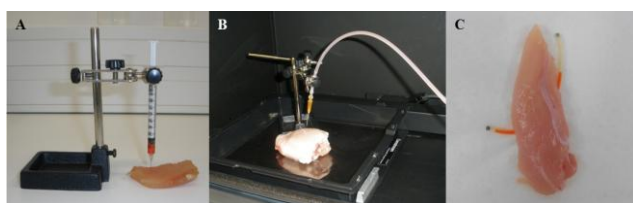
Figure 3. The different applications of the doxorubicin: (A) injection; (B) infusion; (C) tube with doxorubicin.

3.3. Study of doxorubicin fluorescence by CCD detector – effect of muscle tissue

In order to study the effect of muscle tissue (the absorption properties) we used a transparent tube filled with doxorubicin at a concentration of 500 µg/ml dissolved in methanol (Figure 4A, B and C). This approach enabled to prevent leakage of doxorubicin in tissue as well as effortless determination of depth of doxorubicin (Figures 4A, B and C). The tube showed no autofluorescence in the area of wavelengths used for monitoring, it did not absorb this radiation; it was therefore an ideal tool for monitoring doxorubicin in muscle tissue. The tube was filled with doxorubicin by a syringe, closed to prevent leakage and monitored at different tissue depths (1-7 mm). Doxorubicin (500 µg/ml in methanol) in the tube was well detectable up to a depth of 6 mm. The fluorescence intensity was decreasing in average by 116 a.u. per 1 mm of the tissue depth (Figure 4D). Complete disappearance of the signal occurred at a depth of 7 mm.

3.4. Study of the distribution of doxorubicin in muscle tissue by CCD detector

Doxorubicin was applied to the muscle tissue (*m. pectoralis*) and detected by its fluorescence depending on the applied volume, depth of the application and concentration of doxorubicin. During application, a syringe was firmly attached
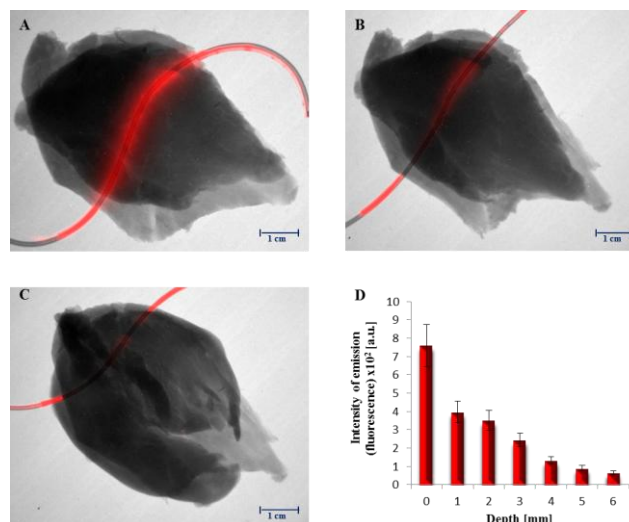

Figure 4. Fluorescence of the doxorubicin (500 µg/ml in methanol) in the tube in different tissue depths: (A) tube in the depth of 4 mm; (B) tube in the depth of 5 mm; (C) tube in the depth of 6 mm; (D) decreasing intensity of fluorescence with the depth of the tube with doxorubicin.

to the stand to ensure the same injection site during repeated applications of doxorubicin into the tissue (Figures 3A and B). Due to inaccuracies of small volumes by dosing syringe, infusion set with a peristaltic pump was chosen for the application. The peristaltic pump provided significantly better repeatability in applied volume (50 µl) of the drug (RSD 5%).

With the increasing amount of the drug (50 – 500 µl; 25 – 250 µg/ml) applied into the tissue, the area of spatial distribution of doxorubicin increases linearly. Significant linear increase in maximum fluorescence intensity of doxorubicin was also recorded. Figure 5A shows distribution maps of doxorubicin fluorescence (500 µg/ml in methanol) applied into the muscle tissue of the same depth of application

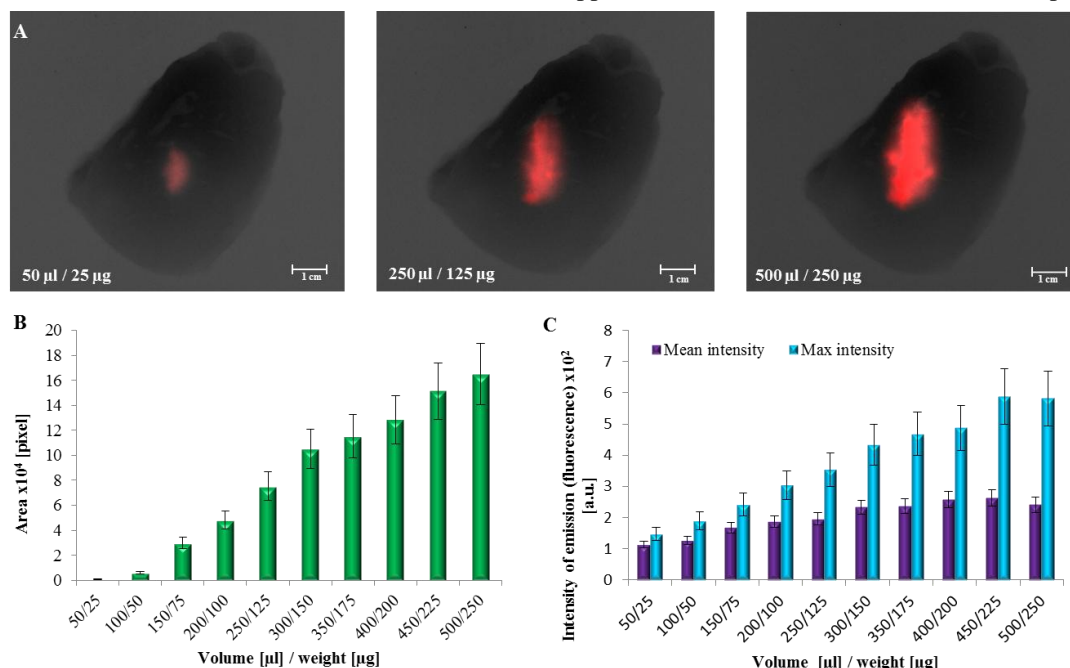**Figure 5.** Fluorescence signal of doxorubicin of increased volume (resp. amount) (500 µg/ml; 50 – 500 µl; 25-225 µg): (A) the doxorubicin in the tissue (50, 250 and 500 µl); (B) the growing area with applied volume of the doxorubicin; (C) the dependence of the intensity of the doxorubicin on the applied volume, average intensity and maximum intensity; conditions as in Figure 2.

(3 mm), in a volume of 50 μl (25 μg), 250 μl (125 μg), and 500 μl (250 μg). It is obvious that the intensity of fluorescence as well as the spatial distribution of doxorubicin increases with the applied volume (amount). Software evaluation of area and intensity of emission provided the information specified in Figures 5B and C. As expected, the area of fluorescence signal increased linearly with the volume (or quantity) of injected drug (Figure 5B). Quantification of a maximum intensity of fluorescence signal showed an increase in fluorescence depending on the amount of the applied drug (Figure 5C). On the other hand, the increase in the average intensity within the area was not so significant in comparison with the maximum signal (average increase 19 a.u./25 μg compared to 55 a.u./25 μg). Furthermore, it has established that after the application of drug into the muscle tissue the spatial distribution occurs almost immediately and a further enlargement of the visible area of the fluorescence signal is not observable even after a long time (three hours), signal intensity is slightly increased due to distribution in the z-axis (downwards, i.e. to the detector) for 3.8%. Doxorubicin was applied to the muscle tissue into different depths (1, 3 and 5 mm) and in these applications poor repeatability of fluorescence intensity of doxorubicin (± 26%) has been determined, especially due to unequal tissue structure.

4. Conclusions

Doxorubicin has good fluorescence properties, which can be used for the detection of fluorescence and observation of this behaviour and distribution in tissues at development of new nanomedical technologies. Doxorubicin fluorescence intensity could be used to monitor the behaviour of this drug in the living organism, but still only to a depth of about 1 cm.

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Cite this article as:

Iva Blazkova *et al.*: **Study of fluorescence of doxorubicinin muscletissue using highly sensitive fluorescence sensing.** *Chemical Sensors* 2014, **4:** 9