PROF. SANTI NONELL (Orcid ID : 0000-0002-8900-5291)

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NanoDCFH-DA: A Silica-based Nanostructured Fluorogenic Probe for the Detection of Reactive Oxygen Species†

Roger Bresolí-Obach[‡†], Luis Busto-Moner[‡†], Claudia Muller[§], Manuel Reina[§], Santi Nonell[‡*]

[‡] Institut Químic de Sarrià, Universitat Ramon Llull, Via Augusta 390, 08017 Barcelona, Spain

[§] Celltec UB, Faculty of Biology, University of Barcelona, Diagonal 643, 08028 Barcelona, Spain

[†]These authors contributed equally to this work.

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*Corresponding author's e-mail: santi.nonell@iqs.url.edu (Santi Nonell)

ABSTRACT

A biocompatible fluorescent nanoprobe for detection of reactive oxygen species in biological systems has been designed, synthesized, and characterized, circumventing some of the limitations of the molecular probe diacetyl 2',7'-dichlorodihydrofluorescein (DCFH-DA). It has been synthetized the nanoparticulate form of DCFH-DA by covalently attaching the widely-used fluorescent probe DCFH-

DA to a mesoporous silica nanoparticle through a linker. The reactivity of nanoDCFH-DA has been tested towards several reactive oxygen species. In addition, it has been proved to slow down DCFH-DA reaction with molecular oxygen and it hampers from interactions with proteins. As final piece of evidence, *in-vitro* studies showed that the nanoprobe is internalized by HeLa cancer cells, thus being capable of detecting intracellularly-generated reactive oxygen species. To sum up, it can be stated that nanoDCFH-DA overcomes two major problems of free DCFH-DA, namely oxidation of the probe by air and interaction with proteins in biological systems. This "nano" approach has thus proven useful to extend the utility of an existing and valuable fluorescent probe to complex biological systems.

INTRODUCTION

Reactive oxygen species (ROS, e.g, hydrogen peroxide (H_2O_2), singlet molecular oxygen ($^1O_2$), superoxide radical anion ($O_2^{\bullet-}$). hydroxyl ($^{\bullet}OH$), alkoxy ($RO^{\bullet}$) and peroxy ($ROO^{\bullet}$) radicals, hypochlorite ion (ClO^-), among others [1]) are highly-oxidant species that participate in a large variety of chemical and biological processes. They are produced by several metabolic processes of aerobic organisms [2] and can react with nucleic acids, lipids or proteins, causing cell damage [3]. When produced in a controlled way, ROS have also been reported to play a vital role in cells, including protein modifications, cell-adhesion regulation and immune system control [4]. The concentration of ROS is controlled by cellular antioxidant defenses, which sometimes can be insufficient or overwhelmed, resulting in accumulation of free ROS inside cells. The imbalance between oxidant and antioxidant species, or oxidative stress, has been related to severe pathologies such as cancer, diabetes or aging [5-7]. In addition to endogenous sources, ROS can also be generated by external stimuli such as light. A prime example is the process of photosensitization [8], which has

important biological and medical applications such as the photodynamic therapy of cancer [9], the photoinactivation of pathogenic microorganisms [10] or the phototoxicity of some drugs [11]. Therefore, the detection of ROS and the mapping of their spatial distribution is of critical importance in biology and medicine [12-14].

Direct, real-time detection of ROS can be done only in a few cases and requires the use of -highly-sophisticated equipment available only in specialized laboratories [15,16]. An alternative simpler strategy is to detect them by chemical trapping methods [17]. Among them, the use of molecular fluorescent probes that change their emission properties upon reaction with ROS is becoming highly popular in connection with the increased use of fluorescence imaging techniques [18,19]. Thus, a number of fluorescent probes have been developed that either show specificity for a given ROS, e.g., Singlet Oxygen Sensor Green (SOSG) for $^1O_2$ [20], dihydroethidium for $O_2^{\bullet-}$ [21] or hydroxyphenyl fluorescein for $^{\bullet}OH$ [22], or are non-specific and can therefore be used to assess oxidative stress [23,24].

2',7'-Dichlorodihydrofluorescein diacetate (DCFH-DA) is arguably the most-widely used non-specific ROS probe (Scheme 1). Its reduced form absorbs in the UV region only and is not fluorescent (see Figure S1 in the Supporting Information file). Upon entering a cell, DCFH-DA is hydrolyzed by esterases to the non-fluorescent 2',7'-dichlorodihydrofluorescein (DCFH), which is oxidized by a variety of ROS to dichlorofluorescein (DCF), a highly conjugated product that absorbs in the visible range and shows intense green fluorescence.

Molecular probes in general and fluorescein derivatives in particular interact with proteins, which affects their cell uptake and response to ROS [25-27]. In order to prevent this problem, encapsulation within nanoparticles or covalent binding to their surface have been proposed and successfully demonstrated [28-30]. In addition, redox ROS probes such as DCFH-DA are slowly oxidized by dissolved molecular oxygen ($^3O_2$), which may lead to unwanted background signals and eventually false positives [13,31]. In this work, a biocompatible silica-based fluorescent nanoprobe

for detection of ROS in biological systems has been designed, synthesized, and characterized, circumventing some of the limitations of the molecular probe DCFH-DA, such as protein complexation and self-oxidation by air. The nanoprobe reactivity has been successfully tested both in phosphate buffer saline solution and inside HeLa cells.

MATERIALS AND METHODS

Materials. 6''-Carboxy-2',7'-dichlorodihydrofluorescein diacetate was supplied by Santa Cruz Biotechnology (Dallas, USA). Hypericin (Hyp) was purchased from HWI Analytik GmbH (Ruelzheim, Germany). Ampicillin, tetraethyl orthosilicate (TEOS), cetyltrimethylammonium chloride solution (25 wt% in H_2O; CTAC), 3-(triethoxysilyl)propyl isocyanate, 4,7,10-trioxa-1,13-tridecanediamine, *N*-(3-dimethylaminopropyl)-*N*'-ethyl-carbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS), sodium hypochlorite solution (NaClO; 140 μM), hydrogen peroxide (H_2O_2, 30% w/v), potassium superoxide (KO_2), methylene blue (MB), Dubelcco's phosphate buffered saline (PBS), L-arabinose and bovine serum albumin (BSA) were purchased from Sigma-Aldrich (St. Louis, USA) and used as received. Absolute ethanol, acetonitrile, trichloroacetonitrile, dichloromethane, hydrochloric acid solution (37 wt% in H_2O; HCl), were supplied by Panreac (Barcelona, Spain). B-PER cell lysis reagent was purchased from Thermo-Fischer (Waltham, USA).

The linker *N*-(4,7,10-trioxa-13-tridecaneamine)-*N*'-(3-(triethoxysilyl)propyl)-urea ($(EtO)_3$-Si-L-NH_2) was synthetized as described in reference [30]. Mesoporous silica nanoparticles (MSNP) and linker-attached mesoporous silica nanoparticles (MSNP-L-NH_2) were synthetized as described in references [30,32].

Dulbecco's modified Eagle's medium (DMEM) with 4.5 g/L glucose, fetal bovine serum (FBS), 200 mM l-glutamine, and penicillin (10,000 units/ml)-streptomycin (10 mg/ml) solution were supplied by Lonza (Basel, Switzerland). Trypsin-EDTA solution and Hanks` Balanced Salt solution with Ca^{2+} and Mg^{2+} ($HBSS^+$) were supplied by Sigma-Aldrich (St. Louis, USA) and WST-1 was supplied by Roche Diagnostics

(Mannheim, Germany). Human HeLa cervix adenocarcinoma cells were from American Type Culture Collection (ATCC CCL-2, Manassas, USA). The cell culture material was supplied by LabClinics S.A. (Barcelona, Spain).

Conjugation of DCFH-DA onto MSNP-L-NH_2. DCFH-DA conjugation to MSNP-L-NH_2 was performed through Steglich amidation adapting the procedure proposed by Steglich *et al.* [33]. Briefly, 4.0 mg (7.5 µmol) of DCFH-DA were previously activated by mixing them for 2 hours with 14.7 mg of EDC (77 µmol) and 14.5 mg (126 µmol) of NHS in 2 mL of dry CH_2Cl_2. The mixture was then added dropwise to 14 mL of a stirred solution of MSNP-L-NH_2 in acetonitrile (7 mg/mL) and the crude was kept reacting for 72 h in darkness and at room temperature. Simultaneously, 0.5 g (5 mmol) of anhydrous Na_2CO_3 were added in order to deprotonate linker's amino terminal groups, since the amidation is not favorable with the protonated form. Afterwards, nanoDCFH-DA was centrifuged and washed 6 times with ethanol (20 min at 13000 rpm).

Physical and chemical characterization of MSNPs. Size and ζ-potential of the synthesized MSNPs were measured using a Nano-ZS Zetasizer equipment (Malvern Instruments LtD, Worcestershire, United Kingdom). For size determination, a 0.1 mg/mL aliquot in ethanol was measured. For ζ-potential examination, a 0.1 mg/mL aliquot in milli-Q water or acidic/basic water was measured.

Infrared spectra of the MSNPs supported on a potassium bromide disk were recorded using a Nicolet Magna 560 FTIR spectrophotometer (ThermoFischer Scientific, Waltham, USA).

Organic elemental analysis of the NPs was carried out in a EURO EA-3000 Elemental Analysis system (Eurovector, Pavia, Italy). The concentration of amino groups (c_{NH_2}) in MSNP-L-NH_2 was determined as the amount of nitrogen in the NPs divided by 3 on account that each linker unit contains 3 nitrogen atoms. Since DCFH-DA contains no nitrogen, comparison the carbon/nitrogen ratio in nanoDCFH-DA relative to MSNP-L-NH_2 allowed to calculate the fraction of amino groups functionalized by the probe and therefore the concentration of DCFH-DA on the nanoparticles.

Sources of ROS. ClO^- and H_2O_2 were added from stock solutions. $O_2^{\bullet-}$ was added as a suspension of solid KO_2 in anhydrous CCl_3CN (140 mM). $^{\bullet}OH$ was generated by UV-A irradiation (353 ± 20 nm; 6.3 mW/cm^2) of a $NaNO_2$ solution (1 mM) [34]. $^1O_2$ was generated by irradiation of a 8.7 µM methylene blue solution with red light (634 ± 8 nm; 7.8 mW/cm^2) [35].

Determination of reactive rate constants. Samples of DCFH-DA or nanoDCFH-DA were exposed to different concentrations of oxidizing agents and the ensuing fluorescence increase was used to calculate the corresponding reactive rate constants. For stable species (ClO^-, $O_2^{\bullet-}$ and ground-state O_2) the method of initial rates was employed. For $^{\bullet}OH$ and $^1O_2$, a comparative method were used instead using terephthalic acid as a reference acceptor for $^{\bullet}OH$ (5.3 µM, rate constant 4.4×10^9 $M^{-1}s^{-1}$) [36] and ADPA as a reference acceptor for $^1O_2$ (1.5 µM, rate constant 8.7×10^7 $M^{-1}s^{-1}$) [37]. Details of the methods are given in Section 1 of the Supporting Information file.

Fluorescence measurements. The fluorescence of the nano- and molecular probes was studied in PBS at the DCFH-DA concentration of 1.5 µM using a Fluoromax-4 spectrofluorometer (Horiba Jobin-Yvon, Edison, USA). Samples were exposed to known amounts of the different ROS after deacetylation by NaOH treatment and subsequent neutralization with sulfuric acid (20 mM).

HeLa cells culture. Human carcinoma HeLa cells were grown as monolayer cultures in DMEM supplemented with 10% (v/v) FBS, 1% (v/v) penicillin-streptomycin solution and 1% (v/v) l-Glutamine. Cells were cultured in an incubator with 5% CO_2 plus 95% air at 37 °C. Cells were seeded in 75 cm^2 flask and subconfluent cell cultures were used.

Viability assay. to evaluate the possible cytotoxicity of nanoDCFH-DA, the viability of HeLa cells was measured by the WST-1 test (Roche Diagnostics, Mannheim, Germany). HeLa cells were seeded in 96-wells plates at 5,000 cells/well for 48h and then exposed to 0.5, 1 and 2 µM of nanoDCFH-DA diluted in $HBSS^+$ for 24h. Finally, the cell culture medium was removed and 100 µL/well of WST-1 (1:10 dilution) was applied. After 2h, absorbance was read at 450 nm.

Cell preparation procedure for microscopical analysis. HeLa cells were seeded in 8-wells plates at 5.000 cells/well for 24h. Then the cell culture medium was removed and 250 µL/well 2 µM nanoDCFH-DA diluted in $HBSS^+$ with and without 10µM Hyp were added and left under incubation at for 2h. The cell culture was washed 3 times with 200 µL/well $HBSS^+$ in order to discard the non-internalized suspended nanoDCHF-DA. Finally, 250 µL/well $HBSS^+$ were added before the microscopical analysis.

Microscopical analysis. All confocal images were acquired using a TCSP SP2 Leica Confocal Microscope (Leica Microsystems CMS, Wetzlar, Germany). Images before and after irradiation were collected using either an Ar-ion laser (488 nm) or a diode-pumped solid state laser (561 nm) and 3 different photomultiplier configurations: transmitted light (for widefield), λ_{obs} 500-550 nm (for DCF), and λ_{obs} 570-620 nm (for Hyp). The samples were irradiated at 561 nm for 2 min in order to generate ROS and, during this time, sequential images were collected. All photographs and videos construction were processed and analyzed using ImageJ1.x software [38,39].

RESULTS

Synthesis and characterization of nanoDCFH-DA

In a previous work, we had identified the structural features of a silica based $^1O_2$ fluorescence nanosensor to optimize its response [30]. It was concluded that mesoporous silica nanoparticles (MSNP) were superior to compact ones and that covalent grafting of the probe to the nanoparticle surface should be through a sufficiently long PEG linker (MSNP-L-NH_2) for optimum performance. The same structural conditions have now been adopted for the preparation of a DCFH-DA-based silica nanoprobe (nanoDCFH-DA) as shown in Scheme 2.

All intermediates obtained in each synthetic step, ranging from MSNP to nanoDCFH-DA, were characterized by their hydrodynamic diameter and ζ-potential by dynamic light scattering techniques (Table S1). ζ-potential was measured under both neutral and acidic conditions to assess the presence of free amino groups at the surface of the MSNPs after each preparation step, since protonation of the amino groups in acidic media results in a more positive ζ-potential. The number of free amino groups was then determined by organic elemental analysis.

Inspection of Table S1 shows that the NP size increases after each preparation step. The final size (300 nm), is appropriate for internalization by mammalian cells such as HeLa [40,41]. Regarding the ζ- potential, the changes observed for MSNP-L-NH_2 upon acidification of the solvent confirm MSNP functionalization with the linker. The observation that the changes are smaller for nanoDCFH-FA indicate that a substantial fraction of the amino groups have successfully reacted with DCFH-DA to form the amide bond. This was further confirmed by infrared spectroscopy: in the 1500-1800 cm^{-1} region, the MSNP spectra show only a band corresponding to adsorbed water bending (1630 cm^{-1}; Figure S2), whereas nanoDCFH-DA shows, in addition, C=O stretching due to the urea moiety (1653 cm^{-1}) and C=C stretching due to DCFH-DA (1560 cm^{-1}) can be observed.

From organic elemental analysis we conclude that the yield of MSNP functionalization with the linker is 0.3 μmol/mg MSNP, a value similar to the one previously published [30]. Likewise, we estimate that 10% of the amino groups are finally functionalized with the probe in nanoDCFH-DA. Figure S3 shows the calculated geometry for nanoDCFH-DA. The average distance between DCFH-DA and the silica surface is estimated to be 1.7 nm. This separation should prevent DCFH-DA from interacting with large biomolecules such as proteins, while at the same time allowing efficient reaction with small species such as ROS.

NanoDCFH-DA activation

As depicted in Scheme 1, the two diacetyl moieties in DCFH should be hydrolyzed in order to efficiently detect ROS. Such activation is performed by esterases in biological environments [42] and can also be achieved in simple solution by basic hydrolysis, e.g., with NaOH, followed by neutralization. This is the procedure followed in our solution experiments. The optimal NaOH concentration and activation time were determined by measuring the increase of fluorescence in nanoDCFH-DA samples upon exposure to ClO^-. Samples prepared under the same conditions without the oxidant were used as references. Table S2 shows the fluorescence ratio between the oxidant-exposed and non-exposed samples. Optimal activation conditions were 1 mM NaOH and 15 minutes contact time before neutralization, under which a maximum fluorescence enhancement of 10.5 was obtained. Detachment of the DCFH-DA or DCFH from the probe by dissolution of the silica matrix during the NaOH treatment was ruled out since the supernatant obtained by centrifugation of the nanoparticles did not show any fluorescence after exposure to oxidants (Figure S4).

Oxidation by molecular oxygen

One problem associated with redox-based probes is oxidation by atmospheric or intracellular $^3O_2$ [13,31]. This can be especially important in long-lasting experiments such as cellular incubations. Figure 1 shows that the fluorescence of DCFH-DA and nanoDCFH-DA indeed increase over time when their solutions are exposed to oxygen in air-equilibrated samples kept in the dark. It can be clearly observed that covalent grafting of the probe to the surface of MSNPs protects it from oxygen. The rate constants for oxidation were deduced from kinetic analysis of the fluorescence intensity vs time plots, using a calibration curve to convert fluorescence intensity data into concentration values (see Section 1 and Figure S5 in the Supporting Information file). The values found,, namely 1.4×10^{-7} M^{-1} s^{-1} for nanoDCFH-DA and 2.0×10^{-6} M^{-1} s^{-1} for DCFH-DA, indicate that the nanoprobe is 14-fold less reactive than its molecular counterpart.

<Figure 1>

Self-sensitized photooxidation

Since nanoDCFH-DA and DCFH-DA are used as fluorescent probes, it is important to ascertain if exposure to light of the deacetylated probes can affect the rate of fluorescence growth. This has been tested by irradiating air-equilibrated samples of the activated probes with green light (524 ± 17 nm; 7.5 mW/cm^2) in PBS solution. The results (Figure 2) show that the fluorescence of the probes increases upon exposure to light in a non-linear fashion. This was probably to be expected since this is essentially an autocatalytic process: Thus, trace amounts of the oxidized form of the probes, formed during exposure to air (Figure 1), may photosensitise the production of ROS, propagating the oxidation and rapidly leading to a dramatic increase in the fluorescence. The process shows saturation, indicating that the originally-reduced probe has been consumed. Figure 2 also shows that the nanoprobe is approximately 10-fold more photostable than its molecular counterpart. It is worth highlighting that typical light doses in microscopy imaging experiments are less than 1 J cm^{-2} [43] at which the nanoprobe has barely been photoconverted.

<Figure 2>

Oxidation by ROS

The reactivity of nanoDCFH-DA and DCFH-DA towards ROS was assessed by exposing them to defined amounts of these species (Figure 3).

<Figure 3>

Both nanoDCFH-DA and DCFH-DA react readily with $^1O_2$, $^{\bullet}OH$, ClO^- and, to a lesser extent, with $O_2^{\bullet-}$, when added at concentrations comparable to those found in biological systems. However, they show different reactivity against each particular ROS (Table 1). Thus, nanoDCFH-DA is less reactive than DCFH-DA against the neutral species $^1O_2$ and $^{\bullet}OH$, but more reactive against the anionic ROS.

The case of H_2O_2 deserves a specific comment since the fluorescence of both nanoDCFH-DA and DCFH-DA increased with the same rate constant (Table 1). Control experiments showed that DCFH-DA was detached from the MSNP surface in the presence of H_2O_2 (Figure S6).

Table 1: Reactive rate constants for DCFH-DA (k^x_{mol}) and nanoDCFH-DA (k^x_{nano}) towards ROS and $^3O_2$ (k^{O2}).

ROS (X)	k^x_{nano} / $M^{-1}s^{-1}$	k^x_{mol} / $M^{-1}s^{-1}$	k^x_{nano} / k^x_{mol}	$(k^x/k^{O2})_{nano}$	$(k^x/k^{O2})_{mol}$	$(k^x/k^{O2})_{nano} / (k^x/k^{O2})_{nano}$
$^3O_2$	1.4×10^{-7}	2.0×10^{-6}	0.35	1	1	1
ClO^-	140	10.5	13	1.0×10^{9}	5.3×10^{6}	189
$O_2^{\bullet-}$	0.31	0.17	1.9	2.2×10^{6}	8.5×10^{4}	26
$^{\bullet}OH$	5.2×10^{7}	2.7×10^{8}	0.19	3.7×10^{14}	1.4×10^{14}	2.6
$^1O_2$	2.2×10^{6}	4.3×10^{6}	0.50	1.6×10^{13}	2.2×10^{12}	7.3
H_2O_2	1.2×10^{-5}	1.2×10^{-5}	1	6.0	6.0	1

Interaction with bovine serum albumin

It has been reported that the interaction of different fluorescent probes with proteins detracts from their performance in biological systems, either because it interferes with cell uptake or leads to fluorescence quenching [25-27]. Figure 4 shows that this is the case for DCFH-DA in the presence of bovine serum albumin (BSA). The fluorescence of its active (oxidized) form shows a clear spectrum

shift and is quenched by more than 85% at 150 µM BSA. In contrast, nanoDCFH-DA shows only a slight broadening of the fluorescence spectrum and with a 25% loss of intensity. Fluorescence lifetime measurements indicate that quenching by BSA is static in nature (Figure S7; see Supporting Information file). This indicates that the MSNPs protect the probe from interaction with BSA.

Using Benesi-Hildebrand plots (Figure S8), the equilibrium constants for BSA binding were calculated as 18 x 10^4 M^{-1} for DCFH-DA and 2.8 x 10^4 M^{-1} for nanoDCFH-DA, 6.4-fold smaller.

<Figure 4>

Intracellular detection of ROS

We studied whether nanoDCFH-DA could be internalized by HeLa cancer cells and detect intracellular ROS. We used hypericin (Hyp) as PS, since it is known to be cell permeant and to generate a variety of ROS upon photoexcitation [44]. Moreover, Hyp can be selectively excited at wavelengths where DCFH-DA does not absorb (e.g., 561 nm) and shows very low absorption where DCF emits, which reduces the risk of inner-filter effects (Figure S9).

Fist we assessed the dark toxicity of nanoDCFH-DA and found it to be negligible below 2 µM even after 24 h of incubation (Figure S10). Cells were then incubated for 2 hours with pre-activated 2 µM nanoDCFH-DA and 10 µM Hyp and were observed by confocal microscopy before and after photoexcitation of Hyp at 561 nm. While no green fluorescence could be observed in the absence of Hyp or before irradiation, a large increase, almost 10-fold, was recorded after exposing Hyp to light (Figure 5). The images show that nanoDCFH-DA is indeed internalized by HeLa cells after 2h incubation, although a fraction remains bound to the cell membrane. Likewise the presence of Hyp inside HeLa cells was confirmed by imaging its red fluorescence (Figure S11).

<Figure 5>

DISCUSSION

A "nano" version of the widely-used generic ROS probe DCFH-DA has been prepared and its reactivity against a variety of ROS has been assessed and compared to that of its molecular counterpart. The first remarkable result is that nanoDCFH-DA is more resistant against $^3O_2$ oxidation (Figure 1), which is advantageous for its use in biological systems as the likelihood of false positives is strongly diminished. The same results are observed for neutral ROS such as $^1O_2$ and $^{\bullet}OH$, while anionic ROS such as ClO^- and $O_2^{\bullet-}$ show higher reactivity against nanoDCFH-DA than against its molecular counterpart (Table 1). These observations are consistent with the surface properties of the nanoprobe. Thus, the water layer adsorbed onto the surface of the nanoparticles is highly structured owing to the presence of silanols [45], making it difficult to diffuse through it. On the other hand, the presence of cationic ammonium groups on the surface at pH 7.4 favors electrostatic attraction of anionic ROS, whereas repulsion dominates in the negatively-charged molecular probe. The last column in Table 1 shows that the combination of reactivity and resistance to $^3O_2$ oxidation makes nanoDCFH-DA better fluorescent probes than molecular DCFH-DA. In addition, nanoDCFH-DA is also 10-fold more resistant to self-sensitized photooxidation (Figure 2). Finally, a further advantage is its lower affinity for proteins, as binding results in severe quenching of the fluorescence. The case of H_2O_2 is special in that exposure to this ROS cleaves the amide bond between probe and nanoparticle [46], releasing it into the external medium. Indeed, the rate constants observed for the nano- and molecular probe are undistinguishable. Fortunately, the very low H_2O_2 physiological concentrations found in cells [47] make this process very slow. The performance of the probe in cells has been tested using HeLa cancer cells. Microscopy imaging shows that the probe is readily internalized by the cells and accumulates in the cytoplasm, where it reacts with ROS generated by

irradiation of hypericin increasing its fluorescence. Contrast values up to 10-fold have been observed.

CONCLUSION

The results of our studies indicate that attaching the generic fluorescent probe DCFH-DA to the surface of mesoporous silica nanoparticle renders a robust nanoprobe that responds to a variety of ROS. The reactivity pattern shows remarkable changes compared to the molecular probe, which reflect the surface properties of the nanoparticle. Higher resistance to oxidation by air and to self-sensitized photooxidation, as well as lower affinity for interaction with proteins, make nanoDCFH-DA a safer and more reliable fluorescence marker for ROS in cells.

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SUPPORTING INFORMATION

Additional Supporting Information is available in the online version of this article:

Section 1. Determination of the reactive rates constants for the different Reactive Oxygen Species tested.

Section 2. Electronic supplementary tables (Tables S1-S2).

Section 3. Electronic supplementary figures (Figures S1-S11).

Section 4. NanoDCFH-DA reactivity towards H_2O_2 (Figure S12).

Section 5. Electronic supplementary references.

REFERENCES

[1] Krumova, K. and G. Cosa (2016) Overview of reactive oxygen species, *Singlet Oxygen Applications in Biosciences and Nanosciences Volume 1*. (Edited by S. Nonell and C. Flors), 1-21. RSC Publishing, London, United Kingdom.

[2] Halliwell, B. (2006) Reactive Species and Antioxidants. Redox Biology Is a Fundamental Theme of Aerobic Life, *Plant Physiol.* **141**, 312–322.

[3] Schieber, M. and N.S. Chandel (2014) ROS function in redox signaling and oxidative stress, *Curr. Biol.* **24**, 453–462.

[4] Ray, P.D., B.W. Huang and Y. Tsuji (2012) Reactive oxygen species (ROS) homeostasis and redox regulation in cellular signaling, *Cell. Signal.* **24**, 981–990.

[5] Birben, E., U.M. Sahiner, C. Sackesen, S. Erzurum and O. Kalayci (2012) Oxidative Stress and Antioxidant Defense, *World Allergy Organ. J.* **5**, 9–19.

[6] Sierra, H., M. Cordova, C.S.J. Chen and M. Rajadhyaksha (2015) Confocal imaging-guided laser ablation of basal cell carcinomas: an ex vivo study, *J. Invest. Dermatol.* **135**, 612-615.

[7] Green, K., M.D. Brand and M.P. Murphy (2004) Prevention of Mitochondrial Oxidative Damage as a Therapeutic Strategy in Diabetes, *Diabetes.* **53**, S110-S118.

[8] Foote, C.S (1991) Definition of Type I and Type II photosensitized oxidation, *Photochem. Photobiol. Sci.* **54**, 659-659.

[9] Agostinis, P., K. Berg, K.A. Cengel, T.H. Foster, A.W. Girotti, S.O. Gollnick, S.M. Hanh, M.R. Hamblin, A. Juzeniene, D. Kessel, M. Korbelik, J. Moan, P. Mroz, D. Nowis, J. Piette, B.C. Wilson and J. Golab (2011) Photodynamic therapy of cancer: an update, *CA Cancer J. Clin.* **61**, 250-281.

[10] Wainwright, M., T. Maisch, S. Nonell, K. Plaetzer, A. Almeida and G.P. Tegos (2017) Photoantimicrobials-are we afraid of the light?, *Lancet Infect. Dis.* **17**, e49-e55.

[11] Boscá, F. and M.A. Miranda (1998) New trends in photobiology (invited review) photosensitizing drugs containing the benzophenone chromophore, *J Photochem Photobiol B.* **43**, 1-26.

[12] Winterbourn, C.C. (2008) Reconciling the chemistry and biology of reactive oxygen species, *Nat. Chem. Biol.* **4**, 278–286.

[13] Kundu, K., S.F. Knight, N. Willett, S. Lee, W.R. Taylor and N. Murthy (2009) Hydrocyanines: a class of fluorescent sensors that can image reactive oxygen species in cell culture, tissue, and in vivo, *Angew. Chem. Int. Ed.* **48**, 299–303.

[14] Wilson, B.C., M.S. Patterson and L. Lilge (1997) Implicit and explicit dosimetry in photodynamic therapy: a new paradigm, *Lasers Med. Sci.* **12**, 182–199.

[15] Jiménez-Banzo, A., X. Ragàs, P. Kapusta and S. Nonell (2008) Time-resolved methods in biophysics. 7. Photon counting vs. analog time-resolved singlet oxygen phosphorescence detection, *Photochem. Photobiol. Sci.* **7**, 1003-1010.

[16] Yu, J., J. Chen, C. Li, X. Wang, B. Zhang and H. Ding (2004) ESR signal of superoxide radical anion adsorbed on TiO_2 generated at room temperature, *J. Phys. Chem. B* **108**, 2781-2783.

[17] Burns, J.M., W.J. Cooper, J.L. Ferry, D.W. King, B.P. DiMento, K. McNeill, C.J. Miller, W.L. Miller, B.M. Peake, S.A. Rusak, A.L. Rose and T.D. Waite (2012) Methods for reactive oxygen species (ROS) detection in aqueous environments, *Aquat. Sci.* **74**, 683–734.

[18] Gomes, A., E. Fernandes and J.L.F.C. Lima (2005) Fluorescence probes used for detection of reactive oxygen species, *J. Biochem. Biophys. Methods.* **65**, (2005) 45–80.

[19] Chen, X., F. Wang, J.Y. Hyun, T. Wei, J. Qiang, X. Ren, I. Shin and J. Yoon (2016) Recent progress in the development of fluorescent, luminescent and colorimetric probes for detection of reactive oxygen and nitrogen species, *Chem. Soc. Rev.* **45**, 2976–3016.

[20] Flors, C., M.J. Fryer, J. Waring, B. Reeder, U. Bechtold, P.M. Mullineaux, S. Nonell, M.T. Wilson and N.R. Baker (2006) Imaging the production of singlet oxygen in vivo using a new fluorescent sensor, Singlet Oxygen Sensor Green®, *J. Exp. Bot.* **57**, 1725–1734.

[21] Nazarewicz, R.R., A. Bikineyeva and S.I. Dikalov (2013) Rapid and specific measurements of superoxide using fluorescence spectroscopy, *J. Biomol. Screen.* **18**, 498-503.

[22] Prince, M., J.J. Reiners, A.M. Santiago and D. Kessel (2009) Monitoring singlet oxygen and hydroxyl radical formation with fluorescent probes during photodynamic therapy, *Photochem. Photobiol.* **85**, 1177-1181.

[23] Keston, A.S. and R. Brandt (1965) The fluorometric analysis of ultramicro quantities of hydrogen peroxide, *Anal. Biochem.* **11**, 1–5.

[24] Chen, X., Z. Zhong, Z. Xu, L. Chen and Y. Wang (2010) 2',7'-Dichlorodihydrofluorescein as a fluorescent probe for reactive oxygen species measurement: Forty years of application and controversy, *Free Radic. Res.* **44**, (2010) 587–604.

[25] Gollmer, A., J. Arnbjerg, F.H. Blaikie, B.W. Pedersen, T. Breitenbach, K. Daasbjerg, M. Glasius and P.R. Ogilby (2011) Singlet oxygen sensor green®: Photochemical behavior in solution and in a mammalian cell, *Photochem. Photobiol.* **87**, 671–679.

[26] Greene, L.E., R. Lincoln and G. Cosa (2017) Rate of Lipid Peroxyl Radical Production during Cellular Homeostasis Unraveled via Fluorescence Imaging, *J. Am. Chem. Soc.* **139**, 15801–15811.

[27] Kalousek, I., D. Jandová and Z. Vodrázka (1980) Electrostatic interactions of fluorescein dyes with proteins, *Int. J. Biol. Macromol.* **2**, 284-288.

[28] Koo Lee, Y.E., R. Smith and R. Kopelman (2009) Nanoparticle PEBBLE sensors in live cells and in vivo, *Annu. Rev. Anal. Chem.* **2**, 57–76.

[29] Ruiz-González, R., R. Bresolí-Obach, Ò. Gulías, M. Agut, H. Savoie, R.W. Boyle, S. Nonell and F. Giuntini (2017) NanoSOSG: a Nanostructured Fluorescent Probe for the Detection of Intracellular Singlet Oxygen, *Angew. Chem. Int. Ed.* **56**, 2885-2888.

[30] Bresolí-Obach, R., J. Nos, M. Mora, M.L. Sagristà, R. Ruiz-González and S. Nonell (2016) Anthracene-based fluorescent nanoprobes for singlet oxygen detection in biological media, *Methods.* **109**, 64–72.

[31] O'Malley, Y.Q., K.J. Reszka and B.E. Britigan (2004) Direct oxidation of 2',7'-dichlorodihydrofluorescein by pyocyanin and other redox-active compounds independent of reactive oxygen species production, *Free Radic. Biol. Med.* **36**, 90–100.

[32] Planas, O., R. Bresolí-Obach, J. Nos, T. Gallavardin, R. Ruiz-González, M. Agut and S. Nonell (2015) Synthesis, photophysical characterization, and photoinduced antibacterial activity of methylene blue-loaded amino- and mannose-targeted mesoporous silica nanoparticles, *Molecules* **20**, 6284–6298.

[33] Neises, B. and W. Steglich (1978) Simple Method for the Esterification of Carboxylic Acids, *Angew. Chem. Int. Ed.* **17**, 522-524.

[34] Takeda, K., K. Fujisawa, H. Nojima, R. Kato, R. Ueki and H. Sakugawa (2017) Hydroxyl radical generation with a high power ultraviolet light emitting diode (UV-LED) and application for determination of hydroxyl radical reaction rate constants, *J. Photochem. Photobiol. A Chem.* **340**, 8–14.

[35] Wilkinson, F., P. Helman and A.B. Ross (1993) Quantum yields for the photosensitized formation of the lowest electronically excited singlet state of molecular oxygen in solution, *J. Phys. Chem. Ref. Data.* **22**, 113-262.

[36] Page, S., W. Arnold and K. McNeill (2010) Terephthalate as a probe for photochemically generated hydroxyl radical, *J. Environ. Monit.* **12**, 1658–1665.

[37] Lindig, B.A., M.A.J. Rodgers and A.P. Schaap (1980) Determination of the lifetime of singlet oxygen in water-d2 using 9,10-anthracenedipropionic acid, a water-soluble probe, *J. Am. Chem. Soc.* **102**, 5590–5593.

[38] Schindelin, J., I. Arganda-Carreras, E. Frise, V. Kaynig, M. Longair, T. Pietzsch, S. Preibisch, C. Rueden, S. Saalfeld, B. Schmid, J.-Y. Tinevez, D.J. White, V. Hartenstein, K. Eliceiri, P. Tomancak and A. Cardona (2012) Fiji: an open-source platform for biological-image analysis, *Nat. Methods.* **9**, 676–682.

[39] Schneider, C.A., W.S. Rasband and K.W. Eliceiri (2012) NIH Image to ImageJ: 25 years of image analysis, *Nat. Methods.* **9**, (2012) 671–675.

[40] Verma, A. and F. Stellacci (2010) Effect of surface properties on nanoparticle-cell interactions, *Small* **6**, 12–21.

[41] Brigger, I., C. Dubernet and P. Couvreur (2012) Nanoparticles in cancer therapy and diagnosis, *Adv. Drug Deliv. Rev.* **64**, 24–36.

[42] Brandt, R. and A.S. Keston (1965) Synthesis of Diacetyldichlorofluorescin: a stable reagent for fluorometric analysis, *Anal. Biochem.* **11**, 6–9.

[43] Wagner, M., P. Weber, T. Bruns, W.S.L. Strauss, R. Wittig and H. Schneckenburger (2010) Light dose is a limiting factor to maintain cell viability in fluorescence microscopy and single molecule detection, *Int. J. Mol. Sci.* **11**, 956-966.

[44] Agostinis, P., A. Vantieghem, W. Merlevede and P.A.M. de Witte (2012) Hypericin in cancer treatment: more light on the way, *Int. J. Biochem. Cell Biol.* **34**, 221–241.

[45] Slowing, I.I., J.L. Vivero-Escoto, B.G. Trewyn and W.S.Y. Lin (2010) Mesoporous silica nanoparticles: structural design and applications, *J. Mater. Chem.* **20**, 7924-7937.

[46] Gómez-Reyes, B. and A.K. Yatsimirsky (2003) Kinetics of amide and peptide cleavage by alkaline hydrogen peroxide, *Org. Lett.* **5**, 4831–4834.

[47] Halliwell, B., M.V. Clement and L.H. Long (2000) Hydrogen peroxide in the human body, *FEBS Lett.* **486**, 10–13.

FIGURE CAPTIONS

Scheme 1. Oxidation of DCFH-DA by ROS.

Scheme 2. NanoDCFH-DA synthesis. Reagents and conditions (a): CTAC, EtOH, H_2O, NH_3, 80 ^oC, 12 min, then HCl (37%), reflux, 24 h. (b): *N*-(4,7,10-trioxa-13-tridecaneamine)-*N*'-(3-(triethoxysilyl)propyl)-urea, EtOH, rt, 24 h. (c): DCFH-DA, EDC, NHS, dry CH_2Cl_2, rt, 2h; then addition of the activated DCFH-DA to MSNP-L-NH_2 in CH_3CN, rt, 72h.

Figure 1. Fluorescence enhancement of DCFH-DA (blue) and nanoDCFH-DA (red) upon oxidation by $^3O_2$ in air-equilibrated PBS (λ_{exc} 490 nm; λ_{obs} 530 nm).

Figure 2. Fluorescence enhancement of DCFH-DA (blue) and nanoDCFH-DA (red) upon green light irradiation (524 ± 17 nm) in air-equilibrated PBS (λ_{exc} 490 nm; λ_{obs} 530 nm).

Figure 3. Determination of the rate constants for reaction of nanoDCFH-DA (red) and DCFH-DA (blue) with different ROS (see Section 1 of the Supporting Information file for details). (a) Fluorescence intensity increase after reaction with $^1O_2$ generated by irradiation of MB with red light. (b) Fluorescence intensity increase after reaction with $^{\bullet}OH$ generated by irradiation of $NaNO_2$ with UVA light. (c) Initial rate of DCF formation after ClO^- addition. (d) Initial rate of DCF formation after $O_2^{\bullet-}$ addition.

Figure 4. Effect of BSA on the fluorescence spectra of DCFH-DA (left) and nanoDCFH-DA (right); λ_{exc} = 490 nm. Insets: fluorescence spectral shifts. Top: Relative change in fluorescence maximum intensity upon BSA addition.

Figure 5. Images of Hela cells incubated with 2 μM nanoDCFH-DA and 10 μM Hyp (top), 2 μM NanoDCFH-DA (middle) and 10 μM Hyp (bottom). Widefield, nanoDCDH-DA fluorescence before irradiation and nanoDCFH-DA fluorescence enhancement after 2 min of irradiation (λ_{irr} = 561 nm) are displayed for each condition. Open bars represent the mean and the standard deviation for thirteen independent cells where only the mean pixel intensity of the images is considered.