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Abstract

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Reference


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Deep UV generation and direct DNA photointeraction by harmonic nanoparticles in labelled samples

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A biophotonics approach based on the nonlinear optical process of second harmonic generation is presented and demonstrated on malignant human cell lines labelled by harmonic nanoparticles. The method enables independent imaging and therapeutic action, selecting each modality by simply tuning the excitation laser wavelength from infrared to visible. In particular, the generation of deep ultraviolet radiation at 270 nm allows direct interaction with nuclear DNA in the absence of photosensitizing molecules.

1 Introduction

We demonstrate here a diagnostic and therapeutic protocol based on the nonlinear optical process of non phase-matched second harmonic (SH) generation by non-centrosymmetric nanoparticles, referred to in the following as harmonic nanoparticles (HNPs). To date, the capability of these recently introduced nanometric probes of doubling any incoming frequency has not been employed for therapeutic use, although it presents several straightforward advantages, including (i) the possibility of direct interaction with DNA of malignant cells in the absence of photosensitizing molecules, (ii) fully independent access to imaging and therapeutic modalities, and (iii) the complete absence of risk of spontaneous activation by natural or artificial light sources other than pulsed femtosecond lasers. Given the unconstrained tunability of the HNP nonlinear conversion process, this approach can be extended to selectively photo-activate molecules at the surface or in the vicinity of HNPs to further diversify the prospective therapeutic action.

Here we show that by tuning the frequency of ultrashort laser pulses from infrared (IR) to visible, SH generation leads respectively to diagnostics (imaging) and therapy (localized phototoxicity). Specifically, we report in situ generation of deep ultraviolet (DUV) radiation (270 nm) in human-derived lung cancer cells treated with bismuth ferrite (BiFeO₃, BFO) HNPs upon pulsed laser irradiation in the visible spectrum, at 540 nm. We observe and quantify the appearance of double-strand breaks (DSBs) in the DNA and cell apoptosis, in the area of the laser beam. We show that DNA damage is dependent on irradiation-time, laser intensity, and NP concentration. We observe that apoptosis and genotoxic effects are only observed when visible light excitation is employed, being almost completely absent when IR excitation is used for imaging.

HNPs, a family of NPs specifically conceived for multi-photon imaging, were introduced in 2005 with the aim of complementing fluorescence imaging labels. Although comparatively less bright than quantum dots, HNPs possess a series of advantageous optical properties, including the complete absence of bleaching and blinking, spectrally narrow emission bands, fully coherent response, and UV to IR excitation wavelength tunability. These unique characteristics have been recently exploited in demanding bio-imaging applications including regenerative medicine research. The possibility of working with long wavelengths presents clear advantages in terms of tissue penetration, as in the IR spectral region, imaging depth is strongly increased by reduced absorption (provided that water absorption bands are avoided) and weak scattering (preventing degradation of spatial and temporal laser profiles).

2 Materials and experimental methods

2.1 Multi-photon imaging

The imaging set-up is based on a Nikon A1R-MP inverted microscope coupled with a Spectra-Physics Mai-Tai DeepSee tunable Ti:sapphire oscillator (Fig. 2a). A Plan APO 40× WI N.A.
1.25 objective was used to focus the excitation laser and to epitroch the nonlinearly excited signal (SH and membrane dye fluorescence). Four independent non-descanned detectors acquire in parallel the signal spectrally filtered by four tailored pairs of dichroic mirrors and interference filters (SH filter; 395 ± 11 nm, Semrock). Optimal pulse compression at the focal plane was adjusted by maximizing the SH signal of individual HNPs dispersed on a coverslip.

2.2 Visible laser irradiation
For visible irradiation experiments, we employed a two-stage non-collinear optical parametric amplifier (TOPAS White, Light Conversion) set at 540 nm (Fig. 2c). The output pulse characteristics are: 30 fs pulse duration, 15 mW average power, 1 kHz repetition rate. For NIR irradiation at 780 nm, the direct output of a Ti:sapphire amplifier (Coherent Duo Elite CEP) adjusted to yield the same energy, pulse duration, and repetition rate of the visible irradiation experiment was used (Fig. 2b). The sample was exposed for 30, 60 and 120 s using a laser spot size (measured by a high resolution beam profiler) of 170 µm or 400 µm diameter obtained by displacing a plano-convex $f = 250$ mm lens.

As for any nonlinear process, the relevant parameter is the pulse peak power at the sample. Due to the very short duration of the pulse employed in this study, the peak power is as high as 2.2 TW cm$^{-2}$ or 400 GW cm$^{-2}$ for a spot size diameter of 170 µm and 400 µm, respectively. The pulse energy is 15 µJ per pulse in both cases. These values can be compared with those reported by Le Harzic et al., who determined cell damage threshold for multi-photon excitation at 517 nm to be above 10 TW cm$^{-2}$ or $4 \times 10^{14}$ mW cm$^{-2}$.

During irradiation, cells were kept at controlled temperature (37 °C), CO$_2$ concentration (5%) and humidity in a portable incubator (Okolab UNO).

2.3 HNP dispersion and characterization
BFO NPs were provided by the German company FEE under a research agreement at high concentration in ethanol. NPs were diluted 1 : 50 in 500 mL ethanol and decanted for 10 days. The supernatant was then taken, ethanol was evaporated, and NPs were re-suspended in distilled water. Successively, NPs were dispersed in an ultra-sonic bath for 24 h and quantified by the Prussian blue assay. For this assay, 50 µL of BFO solution were diluted in 50 µL HCl 6 M and 100 µL of 5% potassium hexacyanoferrate (Sigma-Aldrich) in PBS were added for 15 min. After incubation, the solution absorbance was measured at 690 nm in a multiwell-plate reader (Synergy HT, BioTek) and compared with the absorbance of a calibration curve with known BFO concentration. BFO NPs were finally diluted to 2 mg mL$^{-1}$ in water. DLS and zeta-potential measurements were carried out with a Malvern NanoZ, yielding: zeta potential $-52.7 ± 3.5$ mV, mean hydrodynamic diameter 165.3 ± 24 nm.

BFO is unanimously considered a very efficient frequency doubler although presently there is no clear consensus in the literature regarding the absolute values of its second-order susceptibility tensor elements. For an order of magnitude, Kumar et al. have measured for $d_{32}$ (the largest tensor element) $298 \text{ pm V}^{-1}$ with 800 nm excitation in a bulk crystal, while Haijsmaier et al. have reported a $d_{32}$ value of 18.7 pm V$^{-1}$ in thin films.

2.4 Cell cultures
Human lung-derived A549 and HTB-182 cancer cell lines are available from ATCC (American Tissue Culture Collection). A549 were grown in Dulbecco’s Modified Eagle Medium (DMEM) containing 4.5 g L$^{-1}$ glucose, 10% heat-inactivated fetal calf serum (FCS) and penicillin/streptomycin (PS) (all cell culture reagents were obtained from Invitrogen). HTB-182 were grown in complete Roswell Park Memorial Institute (RPMI) 1640 medium (Invitrogen) supplemented with 10% FCS and PS.

2.5 Cell staining
Cells were grown for 24 h and BFO HNPs at 50 µg mL$^{-1}$ were added for further 24 h, then cell layers were fixed in 4% formaldehyde in PBS, permeabilized for 5 min in 0.1% Triton X-100 (Sigma-Aldrich) in PBS and exposed to 5 µg mL$^{-1}$ of FM1-43FX fluorescent probe (Invitrogen, 1 mg mL$^{-1}$ stock solution in DMSO) for 1 min on ice, washed with PBS and maintained in 4% buffered formaldehyde at 4 °C until the acquisition of images.

2.6 Determination of cytotoxicity
The cytotoxic effect of BFO HNPs at 100 µg mL$^{-1}$ was determined after 5, 24 and 72 h incubation by the MTT assay, as previously described. Briefly, after incubation with the BFO or vehicle, MTT solution (3-(4,5-dimethyl-2-thiazoyl)-2,5-diphenyltetrazolium bromide (Sigma-Aldrich) 5 mg mL$^{-1}$ in PBS) was added to cells for 2 h. Then, the cell culture supernatants were removed, the cells layers were dissolved in 2-propanol/0.04 N HCl and absorbance at 540 nm was measured using a multi-well plate spectrophotometer (Synergy HT, BioTek). Experiments were conducted in triplicate, repeated twice and expressed as cell survival compared to cells exposed to vehicle. Means ± standard deviations were calculated.

2.7 Dithiothreitol assay
BFO HNPs were diluted at 25 µg mL$^{-1}$ or 100 µg mL$^{-1}$ in nanopure water containing 250 µM DTT (Sigma-Aldrich) and incubated for 30 min at 37 °C, then 90 µL of Ellman solution (5 mM 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), Sigma-Aldrich) were added and the absorbance was measured at 405 nm using a multiwell-plate reader (Synergy HT). DTT concentration was calculated by comparison with a standard DTT solution. Experiments were conducted in triplicate wells, repeated twice and converted as % of consumed DTT. Means ± standard deviations were calculated.
2.8 Determination of reactive-oxygen species production

Two different methods were used to determine reactive oxygen species (ROS) production in cells. In the former, ROS were detected by measuring the oxidation of DHE to ethidium. Cells were grown for 24 h and BFO at 25 \( \mu \text{g mL}^{-1} \) or 100 \( \mu \text{g mL}^{-1} \) were added for further 24 h. Then, cell layers were washed with PBS and 100 \( \mu \text{M} \) DHE (Sigma-Aldrich) in RPMI 1640 was added for 15 min at 37 \( ^\circ \text{C} \). Cells layers were further washed and lysed with 0.1% Triton X-100 (Sigma-Aldrich) in PBS. Ethidium production was determined using a fluorescent multiwell-plate reader (Synergy HT) at \( \lambda_{\text{ex}}/\lambda_{\text{em}} = 485/580 \text{ nm} \).

The second ROS measurement method involved the use of DCFH-DA. DCFH-DA is trapped inside cells as a sensitive cytosolic marker of oxidative stress, since its oxidation leads to the formation of the fluorescent dichlorofluorescein (DCF). Cells were grown and treated with BFO as previously described, then cells layers were washed with PBS and exposed to 20 \( \mu \text{M} \) DCFH-DA in Hank’s buffer solution (HBSS) (both from Invitrogen) for 40 min at 37 \( ^\circ \text{C} \). After incubation, the cell layers were further washed and fluorescence of DCF was measured at \( \lambda_{\text{ex}}/\lambda_{\text{em}} = 485/527 \text{ nm} \). Experiments were conducted in triplicate, repeated twice and expressed as fold-increase compared to cells exposed to vehicle. Means ± standard deviations were calculated.

2.9 Immunohistochemistry

Fixed cell layers were washed twice with PBS, permeabilized with cold methanol (–20 \( ^\circ \text{C} \)) for 5 min, washed with PBS and incubated for 10 min at RT in 3% \( \text{H}_{2}\text{O}_{2} \) in methanol. Then, cells were washed and incubated for 1.5 h at RT with anti-phospho-histone H2AX (Ser139) or anti-cleaved PARP antibodies (both from Cell Signaling) diluted 1 : 250 in Dako REALTM Antibody Diluent (Dako). After incubation, cell layers were washed twice with PBS and incubated with undiluted anti-rabbit HRP-conjugate antibody (EnVision+ Sytem, Dako) for 30 min at RT. Cells were further washed and HRP activity was revealed using the DAB+ CHROMOGEN system from Dako, according to the supplier instructions. Finally, cells were counterstained with hematoxylin and images were taken using a microscope (DM IL LED from Leica) equipped with a digital camera (ICC50HD, Leica). Three pictures per treatment were taken and positive cells in a surface of 0.3 mm\(^2\) around the laser-spot were counted using ImageJ software. Experiments were conducted in triplicate wells, repeated twice and expressed as % of positive cells per picture.

2.10 Statistical analysis

Data were compared using a homoscedastic, two-tailed distributed Student’s \( t \)-test. Details about comparisons are specified in the caption of each figure. Significance is expressed as: \( \dagger p > 0.05; \ast p < 0.05; \ast\ast p < 0.01; \ast\ast\ast p < 0.001 \).

3 Results

As an example of HNP based imaging, Fig. 1 displays lung-derived A549 cancer cells stained with FM1-43FX cell membrane dye exposed for 5 h to BFO HNPs at 50 \( \mu \text{g mL}^{-1} \). The image was acquired upon near IR excitation at 790 nm, the two-photon excited fluorescence from the dye is shown in yellow, while the intense blue spots correspond to SH radiation emitted by HNPs. The latter have the tendency to remain attached to cell membranes without being internalized due to their relatively large size. As for other nanobiotechnological approaches, selective binding of NPs to specific cell membrane receptors would rely on the presence of targeting molecules at their surface, a strategy that was not implemented in this exploratory study.

![Fig. 1 Multiphoton imaging of a HNP treated sample. Lung-derived A549 cancer cells exposed for 5 h to 50 \( \mu \text{g mL}^{-1} \) BFO HNPs. Yellow: two photon excited fluorescence from cell membrane dye FM1-43FX. Blue: SH signal from HNPs. Scale bar: 10 \( \mu \text{m} \).](image)

![Fig. 2 Different experimental configurations. (A) multiphoton microscope imaging; (B) NIR irradiation; (C) visible irradiation.](image)
3.1 BFO cytotoxicity and ROS production

Given the novelty of the nanomaterial employed in this work, prior to the assessment of photo-therapeutic modality, BFO HNPs were characterized and screened for biocompatibility in terms of cytotoxicity and oxidative effect. As reported in Fig. 3 (MTT), the cytotoxic effect of 100 μg mL⁻¹ BFO HNPs was assessed after 5, 24 and 72 h exposure on two lung-derived cell lines (A549, HTB-182). BFO cytotoxicity was found acceptable in both samples as HNPs did not cause any detectable effect on cell survival after 5 h exposure, and after 24 h and 72 h cell viability remains remarkably high (>75%), comparable to that observed with HNPs composed of other nanomaterials previously screened.¹⁵

In the present work, it is of paramount importance to precisely quantify ROS production by NPs to identify their possible role in the therapeutic protocol described below. The catalytic activity of BFO HNPs was first measured in a cell free environment by the dithiothreitol (DTT) assay.¹⁹ The histogram of Fig. 3 (DTT) indicates that BFO HNPs do indeed show a dose-dependent consumption of DTT after 1 h incubation, suggesting that they can exert catalytic production of superoxide. On the ground of this finding, we determined the ROS production by BFO HNPs in cell cultures using two fluorescence assays: dihydrothidium (DHE) and-carboxy-dichlorodihydrofluorescein diacetate (DCFH-DA).¹⁶,²⁰ We could detect a dose-dependent increase of ROS, more pronounced in A549 cells than in HTB-182 (Fig. 3 DHE and DCFH-DA). We point out that ROS production remains however low compared to that induced by other metal-based NPs,¹¹,¹⁸,²⁶,²¹ for a quick comparison, human fibroblasts exposed for 2 h to 25 μg mL⁻¹ of Ag nanoparticles yield a three-fold increase in the DHE output, similar to the positive control (H₂O₂) tested by AshaRani et al.²⁰ Overall, the result of this thorough screening indicates a good biocompatibility of this nanomaterial, tested for the very first time for biological applications, and sets the ground for the light-triggered HNP–cell interaction described in the following.

3.2 Laser irradiation experiments

DNA absorption is particularly efficient in the deep UV (DUV, <300 nm), as all DNA bases possess absorption bands peaking around 260 nm with negligible intensity from 310 nm. Irradiation of cell cultures at this wavelength results in DSBs and evokes a complex network of molecular responses, eventually resulting in DNA repair and/or cell apoptosis.²²–²⁴ In our work, these two different biological phenomena were monitored performing two ad hoc bioassays: γH2AX and cPARP, respectively.

Histone variant H2AX is a key component of the early stage response to DNA damage, as upon UV exposure it is phosphorylated at its carboxyl terminus to form γH2AX at the DSB sites.²²,²³,²⁵,²⁶ After the first appearance of UV-induced DNA-damage, cells first activate DNA-repair mechanisms and then apoptosis occurs to eliminate potentially hazardous cells. UV-dependent apoptosis is caused by the activation of caspase-3 and subsequent cleavage of the poly (ADP-ribose) polymerase (PARP), resulting in a cleaved-form (cPARP) with a mass of 89 kDa.²⁶–²⁸

For the irradiation experiment, cells were plated in 35 mm Petri dishes with a glass bottom for 48 h, then medium was replaced and cells were incubated for 24 h with BFO HNPs (25 or 100 μg mL⁻¹, 2 mg mL⁻¹ stock solution in water) or a negative control containing the vehicle (distilled water). The sample was exposed for 30, 60, or 120 s to ultrashort (30 fs) pulses of visible light with a laser spot size of 170 μm diameter. During irradiation, cells were kept under stable conditions in a portable incubator. After light treatment, cells were incubated for 30 min (γH2AX assay) or 24 h (cPARP assay)²⁵,²⁹,³⁰ and then fixed with 3% formaldehyde in PBS. Frequency doubling of femtosecond pulses of visible light (540 nm) by HNPs attached to cell membranes generates DUV photons in the close vicinity of cell nuclei, optimally placed for direct photo-interaction (see Fig. 1). The biological effects of such laser irradiation are reported in the immunohistochemistry (IHC) images of Fig. 4. The two columns are associated with the two human malignant cell lines already tested for cytotoxicity, A549 and HTB-182. The control samples (first row) show no expression for both reporters, confirming that they are not present under physiological conditions, while the clear effect (positive cells in brown) visible in rows two and three for treated (exposed to HNPs and laser) cells indicates that a strong interaction upon irradiation...
observe that BFO HNPs at 25 and 100 \( \mu \text{g mL}^{-1} \) reduce expression of cPARP as compared to those exposed to HTB-182 cancer cell lines untreated (first row) or exposed to 100 \( \mu \text{g mL}^{-1} \) BFO HNPs (2 and 3), incubated for 24 h, and irradiated at 540 nm for 120 s. The expression of cPARP (fourth row) and \( \gamma \text{H2AX} \) (third row) observed by IHC after further 24 h or 30 min of incubation, respectively. Positive cells are indicated in brown, nuclei in blue, and HNP aggregates appear as small brown spots.

Fig. 4 Immunohistochemistry. Human lung-derived A549 and HTB-182 cancer cell lines untreated (first row) or exposed to 100 \( \mu \text{g mL}^{-1} \) BFO HNPs (2 and 3), incubated for 24 h, and irradiated at 540 nm for 120 s. The expression of cPARP (fourth row) and \( \gamma \text{H2AX} \) (third row) observed by IHC after further 24 h or 30 min of incubation, respectively. Positive cells are indicated in brown, nuclei in blue, and HNP aggregates appear as small brown spots.

Fig. 5 Spatial localization of the expression of cPARP and \( \gamma \text{H2AX} \) reporters. Analysis of one representative IHC image (480 × 650 \( \mu \text{m} \)) for the treatments in Fig. 4. For each rectangular (80 × 130 \( \mu \text{m} \)) data region the % ratio of cells positive for the expression of cPARP (column 1) and \( \gamma \text{H2AX} \) (column 2) is expressed in false colors according to the colorbar. Black empty square: laser spot region (170 × 170 \( \mu \text{m}^2 \)).

The quantitative assessment of the effects of \textit{in situ} DUV generation is reported in the comprehensive Fig. 6. In the histograms, the number of IHC-positive cells is expressed as % ratio of total cells in the area of the laser-spot. First, one can observe that BFO HNPs at 25 and 100 \( \mu \text{g mL}^{-1} \) without laser irradiation do not cause any significant increase of cPARP and \( \gamma \text{H2AX} \) expression, ensuring that oxidative and cytotoxic effects of BFO alone do not interfere with irradiation assays.

Upon laser-exposure, the ratio of cells positive for cPARP and \( \gamma \text{H2AX} \) clearly increases in an exposure time-dependent manner. Cells treated with 25 \( \mu \text{g mL}^{-1} \) showed a reduced expression of cPARP as compared to those exposed to 100 \( \mu \text{g mL}^{-1} \), which remains anyway always significantly greater than that measured with vehicle. This decrease in expression is more pronounced for HTB-182 cells. The number of positive cells for \( \gamma \text{H2AX} \) expression decreases in the two cell lines accordingly.

In A549 the expression of the two proteins is comparable, whereas HTB-182 cells express systematically more \( \gamma \text{H2AX} \). Such a stronger DNA-repair related enzymatic activity seems correlated with higher cell viability: the maximal expression of cPARP (apoptosis) is around 60% while in A549 it reaches almost 100%.

To complete the characterization of the HNPs+visible irradiation synergistic effects on cells viability, we further investigated laser intensity and HNP concentration dependence. A549 and HTB-182 cells were exposed to 100 \( \mu \text{g mL}^{-1} \) HNPs or vehicle and irradiated with the same laser average power focused onto a larger surface (400 \( \mu \text{m} \) diameter), corresponding to a neat five-fold intensity decrease with respect to the previous irradiation protocol. As reported in the third and fourth rows of Fig. 6, a substantial decrease in the expression of both cPARP and \( \gamma \text{H2AX} \) was detected in both cancer cell lines. However, the difference between cells treated with BFO and with vehicle remained always significant, except for the expression of cPARP on HTB-182 cells after 30 s exposure, suggesting that BFO NPs generate DUV at lower irradiation intensity as well. The greatest difference between cells exposed to BFO and to vehicle was always observed for the longest exposure and the decrease on cPARP expression was greater compared to the expression of \( \gamma \text{H2AX} \). The dramatic reduction in cPARP is consistent with the square intensity dependence of the second harmonic process at the basis of the NP–cell interaction.

The major decrease in cell viability observed upon irradiation of HNP-treated samples, together with the high spatial localization of the biological effects (Fig. 5), makes HNP-based approaches amenable for developing therapeutic (photodynamic) protocols. To assess the wavelength-dependent multimodality of the method, immunohistochemistry was performed also for imaging only modality. Cells exposed to 100 \( \mu \text{g mL}^{-1} \) were irradiated at 780 nm (SHG at 390 nm, outside the DNA-base
absorption band) with the same pulse intensity and laser repetition rate as for the data presented in the first two rows of Fig. 6. Expression of γH2AX and cPARP was detected according to the protocol previously described. Compared to 540 nm laser excitation, the percentage of positive cells for γH2AX is significantly lower at this longer wavelength, and no expression of cPARP was observed under all exposure conditions. As reported in previous studies, using the HNP approach, imaging is not limited to near infrared wavelengths but it can be performed even above 1.5 μm, with clear advantages in terms of imaging penetration depth (thanks to decreased scattering and absorption)²⁸,²⁹ and decreased phototoxicity.

4 Discussion

The results and control experiments presented altogether confirm that UV generation and related cytotoxic and genotoxic effects can be unambiguously ascribed to BFO HNPs non-linearly excited by ultrashort visible light pulses. Major effects on cell viability (up to 100% apoptosis) are observed in the irradiated areas. The difference on γH2AX expression between cells irradiated with lower laser intensity or exposed to lower particle concentration suggests that the synergistic effect between laser and HNPs is dominated by direct DNA photodamage, but they might also imply other subsidiary mechanisms, suggested in the literature, such as a thermal effect and cell membrane disruption.³¹-³³ The apoptotic cell fraction in samples exposed to the 540 nm laser but not treated with HNPs (cells exposed to vehicle in the histograms in the first and second row of Fig. 6) can be ascribed to direct two-photon absorption by DNA, as previously observed in other studies.³⁴,³⁵

Non HNP-specific interaction can be easily counteracted thanks to the fact that the cellular effects exerted by BFO HNPs are limited to the area of the laser spot, as highlighted in Fig. 5. If IR imaging is preliminarily performed to precisely define the zone needing irradiation, treatment conserves its high specificity. It should be noted that treatment localization is expected to be greater in the proposed HNP-based approach, which is primarily based on direct UV absorption by DNA, than in photodynamic treatments involving direct UV irradiation or upconverting and plasmonic NPs combined with photo-sensitizing drugs. In these latter cases, the NP-cell interaction is essentially mediated by ROS, which are known to diffuse through tissues.³⁶,³⁷

It is worth pointing out that all optical methods listed are limited to surface neoplasms that can be accessed either directly or endoscopically, as optical penetration depth in tissues (strongly depending on the wavelength) remains limited at best to a few millimetres. On the positive side, the proposed approach shares the advantages of nonlinear techniques, enabling to maximize penetration. Moreover, and notably, the activation of the process here is intrinsically limited to femtosecond-pulse excitation and cannot be obtained by any other artificial or natural light source, differently also from the approaches based on sequential up-conversion of light frequency. This purely physical constraint greatly decreases the risk of unspecific treatment activation for surface lesions. Beyond medical approaches, we believe that the proposed approach can be used for biological applications to photo-trigger in vitro or tissues with a high degree of spatial selectivity, for example grafting on HNPs some photo-cleavable molecules or taking advantage of DUV generation for protein cross-linking.

5 Conclusions

In conclusion, we have presented and demonstrated on human-derived cancer cell lines an original nanobiotechnological approach based on the nonlinear optical properties of HNPs. The proposed method enables wavelength-selected imaging and direct DUV generation and photo-interaction with nuclear
DNA. The biocompatibility of BFO, a nanomaterial firstly applied for biological applications to the best of our knowledge, screened for cytotoxicity and generation of oxidative stress, was found comparable to those of other HNPs or metal-based nanoparticles currently used in biomedical studies.\textsuperscript{11,18,20,21} It should be noted that all HNPs, possessing high nonlinear efficiency,\textsuperscript{13} can exert the effects described in this study, which are therefore not unique to BFO.

DSB DNA damage and induction of apoptosis are typical targets of photodynamic therapies, which normally involved the use of direct UV radiation (with poorer tissue penetration and lack of specificity) and/or chemical photosensitizers.\textsuperscript{18,19} To date, NP-based strategies imply using organic sensitizers (with some notable exceptions\textsuperscript{8,9}) and are mediated by ROS generation.\textsuperscript{21,27,41,42} As for classical phototherapy, these approaches can generate major side effects due to the presence of toxic compounds and ROS which can diffuse to nearby tissues generating oxidative stress.\textsuperscript{31,44} The approach proposed, based on the nonlinear optical response by HNPs and direct photo-interaction with nuclear DNA, might avoid side effects due to organic ligands and diffusion of toxic compounds, increasing selectivity and treatment localization. Finally, and very notably, the proposed strategy allows to totally decouple diagnostic modality (IR imaging) from the therapeutic photodynamic action (visible irradiation), by simply tuning the excitation laser wavelength.

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