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Light-sensitive dextran-covered PNBA nanoparticles to continuously or discontinuously improve the drug release

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ABSTRACT

In this work, photo-sensitive core/shell nanoparticles (NPs) based on biocompatible dextran-*g*-poly(*o*-nitrobenzyl acrylate) copolymers (Dex-*g*-PNBA), containing dextran as hydrophilic backbone and PNBA as photosensitive grafts, were formulated using two processes. In the first process (nanoprecipitation), NPs were prepared using preformed Dex-*g*-PNBA copolymers. Using the second process (emulsion/organic solvent evaporation), "clicked" or "unclicked" NPs were obtained carrying out (or not) an interfacial *in situ* click chemistry, respectively. Two model molecules, Nile Red (NR) and Doxorubicin (DOX), were encapsulated and their controlled release from NPs was investigated under UV irradiations to demonstrate the high potential of such photosensitive NPs in biomedicine applications as drug delivery nanocarriers. According to such irradiations, improved release was easily observed. Release kinetics depended on the formulation process and the NPs core chemistry, but not on the occurrence of the interfacial *in situ* click chemistry. More interesting, a stepped release of such model molecules may easily be obtained.

Keywords:

Drug Delivery System; Polysaccharide; Glycopolymer; Photo-responsive polymer; Light-responsive; Light-triggered; Anticancer

I) INTRODUCTION

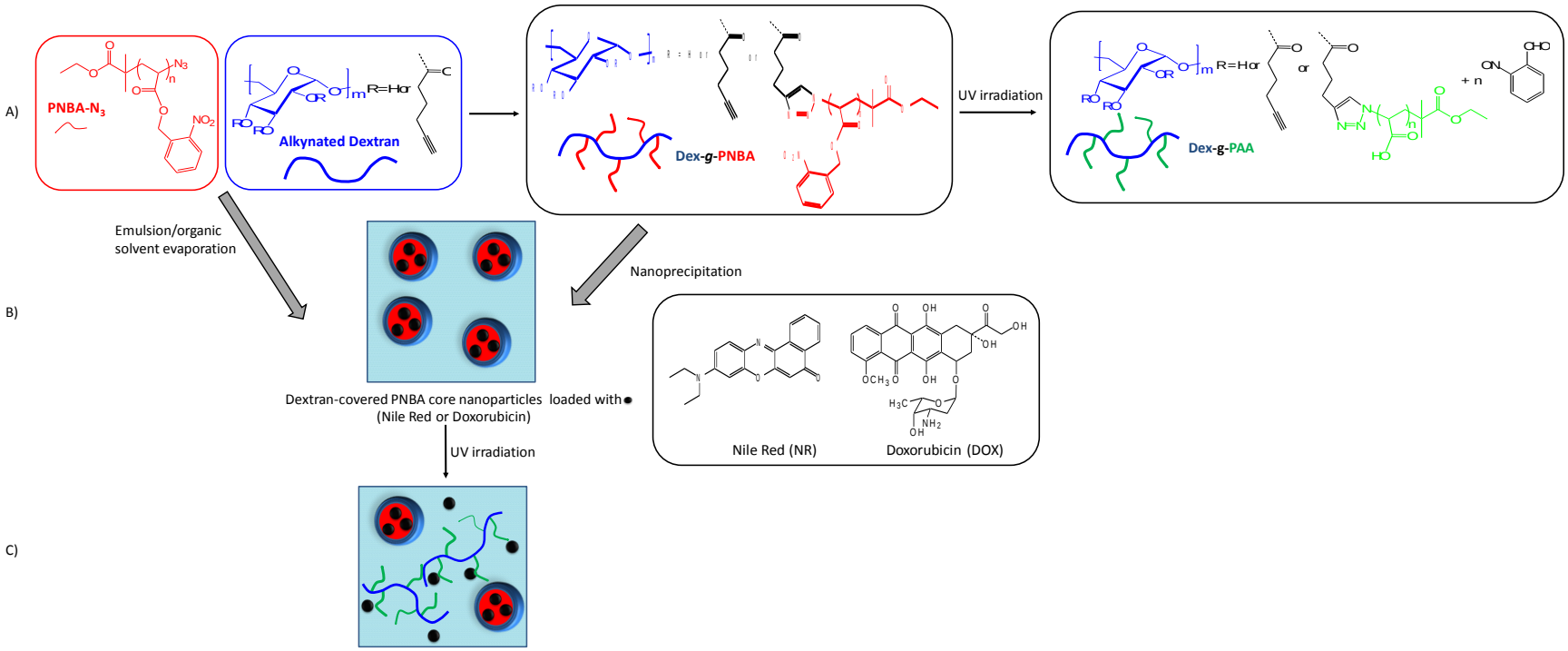
Release from drug delivery systems (DDS) can occur by various mechanisms, including degradation, swelling/shrinkage, or is mainly considered as diffusion driven. Nevertheless, since last decades, smart DDS[1–5] that are sensitive to internal or external stimuli including among others, thermosensitive, pH-sensitive and light-sensitive DDS… have been developed to enhance the treatment efficiency (drug biodistribution and pharmacokinetics). Light as external stimulus appeared very attractive given that emission wavelength, intensity and duration of the irradiation can be modulated in order to control the drug release in both space and time (spatiotemporal control). Indeed, the light-induced release starts when the light is switched on and was demonstrated to be very fast in comparison to other stimuli. This leads to a high drug local concentration, which allows to reduce the overall injected dose. Such light-sensitive DDS are nowadays summarized in several reviews.[6–11]

The on-demand drug release occurs due to a physical/chemical properties change of the photo-responsive moieties into the polymer chains composing the light-sensitive nanoparticles (NPs).[6–12] On one hand, swelling/shrinkage of DDS can be caused by a reversible or irreversible photo-induced crosslinking when coumarin or cinnamic esters are used as side groups, respectively. On another hand, the photo-sensitive polymer chains polarity can be photo-switched from hydrophobic to hydrophilic. For instance, under irradiation, azobenzene or spiropyran can undergo a reversible *E*/*Z* isomerization, 2-diazo-1,2-naphthoquinone may be chemically modified, and coumarinyl, pyrenylmethyl or *o*-nitrobenzyl (NB) esters can be irreversible split-up from the polymer chain. More particularly, NB moieties were introduced as side groups by chemical modification of some polymers [13, 14, 15]. NB was also used as monomer unit during polycondensation/polyaddition,[11,16,17] or *via* (co)polymerization of *o*-

nitrobenzyl-based monomers [18,19,20,21,22] By this later way, the photocleavage of such photochromic part leads to the irreversible breaking of the polymer chains, and thus to the irreversible disruption of DDS.

To the best of our knowledge, only two papers deal with either poly(*o*-nitrobenzyl acrylate) (PNBA) [23] or poly(*o*-nitrobenzyl methacrylate) (PNBM)[24] core NPs. It should be noted that micellization of i) copolymers based on *o*-nitrobenzyl acrylate (NBA) [25–31] or methacrylate[11,30,32] as comonomer units or ii) copolymers with PNBA or PNBM polymeric parts[18,33–38] is the more often reported.

Recently, our team reported the first controlled radical polymerization of NBA using Single Electron Transfer Living Radical Polymerization (SET-LRP)[39] and the synthesis of amphiphilic grafted photosensitive copolymers named Dex-*g*-PNBA.[12] These copolymers based on dextran (Dex) as hydrophilic polysaccharidic backbone and on PNBA as photo-responsive hydrophobic grafts were produced by Huisgen-type Copper(I)-catalyzed Azide–Alkyne Cycloaddition (CuAAC) click-chemistry (Scheme 1A). Dextran-covered PNBA core NPs with an average diameter of 130 nm were reported.[40] On one hand, we demonstrated that such NPs could be disrupted under UV irradiation [40] by photolysis of the hydrophobic PNBA grafts to hydrophilic polyacrylic acid (PAA) ones (Scheme 1C**).** On another hand, such NPs were non-cytotoxic towards Caco-2 (human epithelial colorectal adenocarcinoma) cells, given that 100% of Caco-2 cell viability was conserved after 48 h incubation with NPs. Motivated by these exciting results, we herein firstly present the encapsulation of model molecules in NPs made either by nanoprecipitation or emulsion/organic solvent evaporation (E/E) processes, two common NPs formulation processes.[41] Nile Red (NR, a hydrophobic fluorescent probe)



Scheme 1: A) Synthesis and quantitative photolysis of Dex-*g*-PNBA copolymers. B) Formulation of drug-loaded NPs by either i) nanoprecipitation of Dex-*g*-PNBA or ii) E/E using PNBA-N_3 as hydrophobic polymer and alkynated dextran as water-soluble surfactant. C) Disruption of dextran-covered PNBA NPs leading to a progressive release of the loaded drug.

and Doxorubicin (DOX, an anticancer drug) were selected as model molecules (Scheme 1B). To the best of our knowledge, this paper is the first one dealing with the encapsulation/release of NR or DOX in/from PNBA-based NPs. Secondly, their releases from NPs were investigated depending on the UV irradiation parameters. Finally, modulated stepped release was explored according a discontinuous UV irradiation.

II) EXPERIMENTAL

II.1) Materials

Alkynated dextran, PNBA-Br, PNBA-N_3 and Dex-*g*-PNBA were produced according to our previous paper.[12] In the present paper, Dex-*g*-PNBA copolymers will be named Dex(δ)-*g*-α$PNBA_M$, where δ and α are the number of pending alkynyl groups and of $PNBA_M$ (M is the number average molecular weight) grafts per 100 glucopyranosic units, respectively. In the same manner, Dex-*g*-PMMA [42,43] copolymers are named Dex-*g*-α$PMMA_M$.

Tetrahydrofuran (THF), dichloromethane (DCM), copper bromide (CuBr, 99.9%), triethylamine (NEt_3), terephthalaldehyde, Nile Red (NR, >98 %), ethylenediaminetetraacetic acid (EDTA) were purchased from Sigma-Aldrich and used without further purification. Doxorubicin hydrochloride (DOX, HCl) was purchased from Sigma-Aldrich and converted to free Doxorubicin (DOX) using NEt_3.

II.2) Elaboration of NR-loaded NPs by nanoprecipitation

On one hand, 25 mg of Dex-*g*-PNBA or Dex-*g*-PMMA were dissolved in 5 mL of THF/H_2O mixture (95/5, v/v) during 24 h. On another hand, NR was dissolved in THF (5 mg/mL) then 10 µL of this solution was added to the first copolymer one. The obtained organic phase was then added dropwise (0.1 mL per min) into 10 mL of Milli-Q water under magnetic stirring. After

complete addition, 10 mL of Milli-Q water were added portion-wise to freeze the final dispersion. Finally, the NR-loaded NPs were recovered by centrifugation (15000 rpm, 15°C, 30 min), washed twice by Milli-Q water, and then freeze-dried.

II.3) Elaboration of drug-loaded "unclicked" NPs by E/E

To formulate NR-loaded NPs, 50 mg of alkynated dextran were dissolved in 10 mL of Milli-Q water (DCM saturated). Meanwhile, 25 mg of PNBA-N_3 (or PNBA-Br, which is the precursor of PNBA-N_3) were dissolved in 1 mL of DCM. 10 µL of NR solution in DCM (5 mg/mL) was added to this organic phase, which was finally poured to the aqueous one. The mixture was sonicated (pulsed mode, 46 W, 2 min, ice bath) using a Vibracell 75 model (Bioblock Scientific). DCM was then evaporated at 37 °C for 2.5 h under gentile stirring. The obtained suspension was centrifuged (10000 rpm, 15 °C, 60 min) to collect NPs, which will be named "unclicked" NPs. Such NPs were resuspended in Milli-Q water, centrifuged again to remove both the free alkynated dextran (non-adsorbed) and the unloaded NR, and finally freeze-dried.

A similar protocol was used to formulate DOX-loaded "unclicked" NPs according to some modifications. In such experiment, the organic phase was made by dissolving 25 mg of PNBA-N_3 (or PNBA-Br) and 15 mg of DOX, HCl in 1 mL of DCM. 18 µL of NEt_3 (5 eq. per DOX, HCl) were then added for neutralization, leading to consider 14 mg of DOX in the feed (m_{DOX}).

II.4) Elaboration of drug-loaded "clicked" NPs by E/E

5 mg of CuBr were added to the first emulsion prior the sonication step to carry out the interfacial *in situ* CuAAC click chemistry between PNBA-N_3 and alkynated dextran chains. To remove residual copper after formulation, EDTA (5 eq. per CuBr) was added to the final washed NPs suspension, and left under stirring for 24 h at room temperature. Finally, "clicked NPs" were collected by centrifugation and purified as above.

II.5) Release of NR from NPs

Sample was prepared by dispersing 200 µL of NR-loaded NPs in 1 mL of Milli-Q water and 1 mL of Phosphate Buffer Saline (PBS, KH_2PO_4/Na_2HPO_4, pH = 7). The final NPs dispersion concentration was equal to 0.11 mg/mL. Emission fluorescence spectra of NR were recorded before irradiation, then after UV irradiation at different times to evaluate the NR release.

II.6) DOX encapsulation and release

DOX loading (DL) and encapsulation efficiency (EE) were calculated according to the following equations. Details are given in the supporting information.

$$DL\ (\%) = \frac{\text{weight of loaded DOX}}{\text{Total weight of loaded NPs}} \times 100 \qquad (1)$$

$$EE\ (\%) = \frac{\text{weight of loaded DOX}}{\text{weight of DOX feed}} \times 100 \qquad (2)$$

The profiles of DOX release were investigated by HPLC. In brief, 20 µL of NPs dispersion (1.25 mg/mL) were introduced per well (96-well microplate), then 200 µL of PBS (pH = 7) were added. Immediately after irradiation (irradiation power: 60 mW/cm^2), released DOX amount was estimated by HPCL, then for 48 h, using a calibration curve of DOX solution in PBS (Figure S2).

II.7) Characterizations

^{1}H NMR spectra were recorded on a Bruker Advance 300 spectrometer (300.13 MHz, 25°C) in DMSO-d_6.

Analytic HPLC was performed on a SHIMADZU CTO-20A/Prominence column oven, with LC Solution program Release 1.23 SP1 using a Pursuit C8 150 x 4.6, 5 µm column and a linear gradient of water/acetonitrile eluent (90/10 (v/v) during 20 min, then 100/0 (v/v) during 10 min) with 1 mL/min flow. The detection was performed at 480 nm using a SHIMADZU SPD20AV/Prominence UV/VIS detector.

Dynamic Light Scattering (DLS) of NPs dispersion at low concentration was evaluated using a Malvern High Performance Particle Sizer (HPPS) instrument. 200 µL of NPs suspension were diluted in 2 mL of NaCl aqueous solution (10^{-3} M). The analysis of the average scattering intensity fluctuations during the measurement allows to estimate the NPs' size and the polydispersity index (PDI). The mean diameter Dz (nm) is the so-called Z-average from cumulated analysis, *i.e.* an intensity–average diameter, and was measured three times with deviation remaining below 5 nm.

NR-loaded NPs suspensions (0.11 mg/mL) in PBS/H_2O (50/50, v/v) were irradiated in 1 cm x 1 cm quartz cuvette whereas DOX-loaded NPs suspensions in PBS (0.11 mg/mL) were irradiated in 96-well microplate. An OmniCure® S1000 UV spot cure lamp in the power range of 54–1150 mW/cm^2, equipped with a light guide of 8 mm diameter and a 320–500 nm filter was used.

Emission fluorescence spectra of NR were performed on a Jasco FP-8300 spectrofluorometer with an excitation wavelength equal to 570 nm. NR emission wavelengths were acquired from 580 to 750 nm.

III) RESULTS AND DISCUSSION

III.1) Unloaded nanoparticles

Dextran-covered PNBA NPs were formulated either by nanoprecipitation of preformed Dex-*g*-PNBA or by E/E process carrying out (or not) an interfacial *in situ* CuAAC click chemistry (Scheme 1B). By this last process, either "unclicked" or "clicked" NPs were designed. On one hand, an *in situ* CuAAC occurred at the liquid/liquid interface between the alkyne groups of the polysaccharidic surfactant (alkynated dextran) and the azido function located at the PNBA chains extremity when CuBr was added to the emulsion prior to the sonication step. This leads to

produce amphiphilic Dex-*g*-PNBA copolymers at the liquid/liquid interface, allowing to covalently link the dextran shell to the PNBA core *via* triazole rings ("clicked" NPs). On another hand, "unclicked" NPs were obtained when the formulation was carried out without adding CuBr. With such "unclicked" NPs, the dextran shell was physically adsorbed onto the PNBA core.[40] All NPs batches were characterized by light scattering to determine the Z-average diameter and polydispersity index (Table 1). Additionally, other non-photosensitive NPs based on Dex-*g*-PMMA[42,43] copolymers were prepared by nanoprecipitation for comparison.

As previously reported, all PNBA-based NPs batches exhibited a colloidal stability in the presence of salt. However, 85% desorption of the physically adsorbed dextran shell ("unclicked" NPs) occurred in the presence of SDS (sodium dodecylsulfate, an anionic drastic competitive surfactant). Fortunately, only 4% of desorption was observed in case of "clicked" NPs or of NPs made by nanoprecipitation.[40] Finally, photosensitive property of such PNBA-based NPs was already evaluated under UV light irradiation. [40]

III.2) Release of NR from NPs made by nanoprecipitation

Table 1 shows average diameters of NPs loaded or not with NR, which were formulated by nanoprecipitation. For the same copolymer used, no significant variation of NPs diameters was observed for both batches. This could be explained by the limited quantity of NR encapsulated (10^{-2} mg of NR with 25 mg of Dex-*g*-PNBA or PNBA). Such low quantity of NR was used in order to not exceed the limit of NR solubility in water (1 μg/mL) if 100% of NR is released.[44] NR was selected herein as a hydrophobic fluorescent probe to evaluate the potential of our photosensitive NPs. In hydrophobic environment, NR shows a fluorescence emission around 610 nm, which falls in hydrophilic environment. Consequently, this behavior will be used to evaluate

Table 1. Diameters and PDI of NR-loaded and empty NPs

Run	Process	[(a)] Used polymers	Nile Red	[(b)] Z-Average diameter (nm)	[(b)] PDI
1	Nanoprecipitation	Dex(15)-*g*-14PNBA$_{3,500}$ (F_{PNBA} = 0.75)	NO	118±3	0.080
2	Nanoprecipitation	Dex(15)-*g*-12PNBA$_{9,800}$ ($_{PNBA}$ = 0.85)	NO	185±2	0.040
3	Nanoprecipitation	Dex(15)-*g*-14PNBA$_{3,500}$ (F_{PNBA} = 0.75)	YES	119±2	0.078
4	Nanoprecipitation	Dex(15)-*g*-12PNBA$_{9,800}$ (F_{PNBA} = 0.85)	YES	184±4	0.045
5	Nanoprecipitation	Dex-*g*-15PMMA$_{4,400}$ (F_{PMMA} = 0.76)	YES	144±3	0.090
6	E/E without CuAAC	PNBA$_{7,900}$-Br + alkynated dextran	NO	109±2	0.144
7	E/E without CuAAC	PNBA$_{7,900}$-Br + alkynated dextran	YES	113±4	0.136
8	E/E with CuAAC	PNBA$_{8,100}$-N_3 + alkynated dextran	NO	118±1	0.103
9	E/E with CuAAC	PNBA$_{8,100}$-N_3 + alkynated dextran	YES	120±2	0.111

(a) F_{PNBA} or F_{PMMA} are the weight fractions of PNBA or PMMA in the grafted copolymers, respectively.

(b) Estimated by DLS.

the release of NR from NPs.

Firstly, in order to check on NR is only released by photolysis of PNBA-core and not by diffusion, NR-loaded photosensitive and non-photosensitive NPs were studied (Runs 3 and 5, Table 1). The evaluation of Normalized Fluorescence Intensity [NFI = (fluorescence intensity)$_t$ / (fluorescence intensity)$_{t0}$] of NR-loaded NPs, and of the maximum emission wavelength (λmax) against time were investigated. As shown on Figure S3, NFI remained constant over two weeks when such NPs were dispersed under gentile stirring in dark. λmax remained also constant (around 630 and 609 nm) in case of NPs based on Dex(15)-*g*-14PNBA$_{3,500}$ and Dex-*g*-12PMMA$_{4400}$, respectively (not showed). These stabilities mean that no NR release was occurring by diffusion. λmax of NR in PMMA environment was lower than that in PNBA core according the higher hydrophobicity of PMMA. More particularly, one can observed that λmax value depends on the hydrophobicity of the NPs cores (Figure 1A). Indeed, the increase of the PNBA weight fraction in Dex-*g*-PNBA copolymers (F_{PNBA}) leads to decrease λmax value before irradiation (λmax = 630 and 605 nm for F_{PNBA} = 0.75 and 0.85, respectively).

Secondly, the UV irradiation of NR-loaded NPs was studied (Runs 3-5, Table 1) to light-trigger the release of such fluorescent probe. Arbitrarily, the PNBA-based NPs suspensions were irradiated using a power irradiation of 320 mW/cm² to check the progressive NPs disruption. In case of Dex-*g*-PMMA based NPs, no variation of the λmax was observed given the PMMA photostability (Figure 1A). Figure 1B shows an example of the evolution of the fluorescence emission spectra of NR-loaded NPs based on Dex(15)-*g*-14PNBA$_{3,500}$ (Run 3, Table 1) recorded at several irradiation times. On one hand, a little shift of λmax can be observed after 30 s of

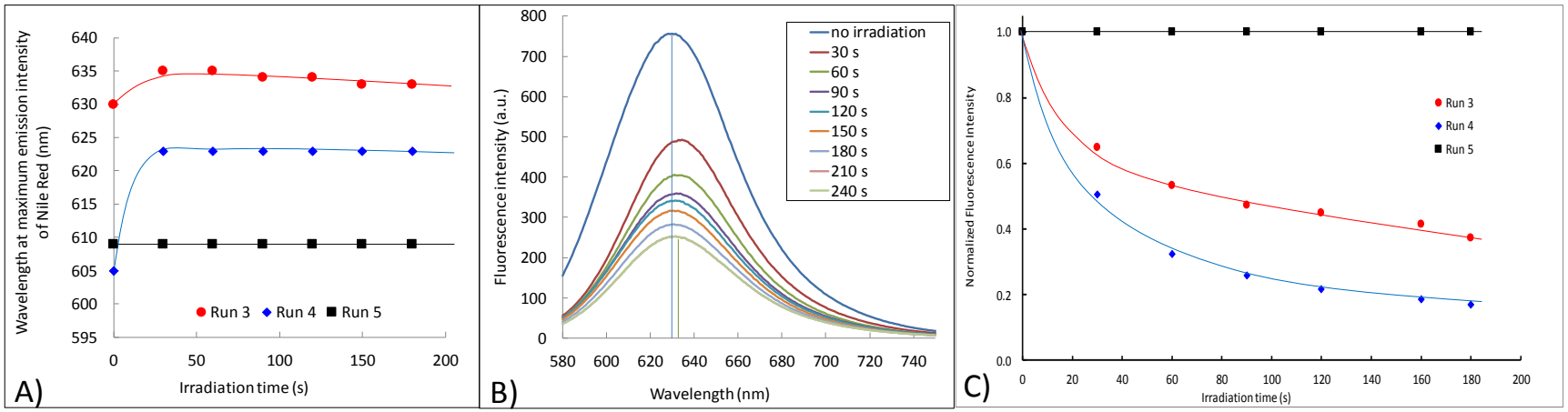


Figure 1. A) Wavelength at maximum emission intensity (λmax) of NR encapsulated in NPs against irradiation time. (Runs 3-5, Table 1). B) Fluorescence emission spectra of NR-loaded NPs based on Dex(15)-*g*-14PNBA$_{3,500}$ (Run 3, Table 1). C) Normalized fluorescence intensity of NR-loaded NPs at λmax *versus* irradiation time (Runs 3-5, Table 1). Irradiation power = 320 mw/cm², λ_{exc} = 570 nm.

irradiation. On another hand, a progressive decrease of the emission fluorescence intensity was observed with the increase of the irradiation time, given the NR fluorescence quenching in water (polar environment). Finally, the evolution of NFI, which was mainly depending on the NR concentration in NPs, was drawn against irradiation time (Figure 1C). A decrease of the NFI with increasing irradiation time was observed due to the progressive light-triggered disruption of PNBA-based NPs. Indeed, due to UV irradiation, PNBA grafts of the Dex-*g*-PNBA are photolyzed to PAA ones (Scheme 1A) as previously demonstrated.[12] Consequently, PAA grafts contain some NBA monomer units in case of partial photolysis, and dextran-*g*-P(NBA-co-AA) are progressively formed inducing a decrease of the NPs core hydrophobicity and a progressive NR release in PBS medium (high polarity). On opposite way, no decreasing of NFI was observed in case of PMMA-based NPs, according to their photostability (not shown).

III.3) Modulation of the NR release kinetics from NPs made by nanoprecipitation

As the UV irradiation power was proved to influence the NPs disruption kinetics,[40] the effect of this parameter to control the NR release was firstly assessed. In the case of PNBA-based NPs (Runs 3-4, Table 1), the evolution of the NFI *versus* the irradiation time were drawn (Figure 2A) depending the irradiation power. In agreement with previous results (Figure 1), the decrease of NFI was observed until reaching one plateau corresponding to the NR fluorescence in PBS/H_2O phase, containing also total (or not) photolyzed copolymers (Dex-*g*-PAA or Dex-*g*-P(AA-co-NBA)). One can easily observed that the NR release kinetics was depending on the irradiation power and was faster with increasing the F_{PNBA} of Dex-*g*-PNBA, thus the hydrophobicity of the

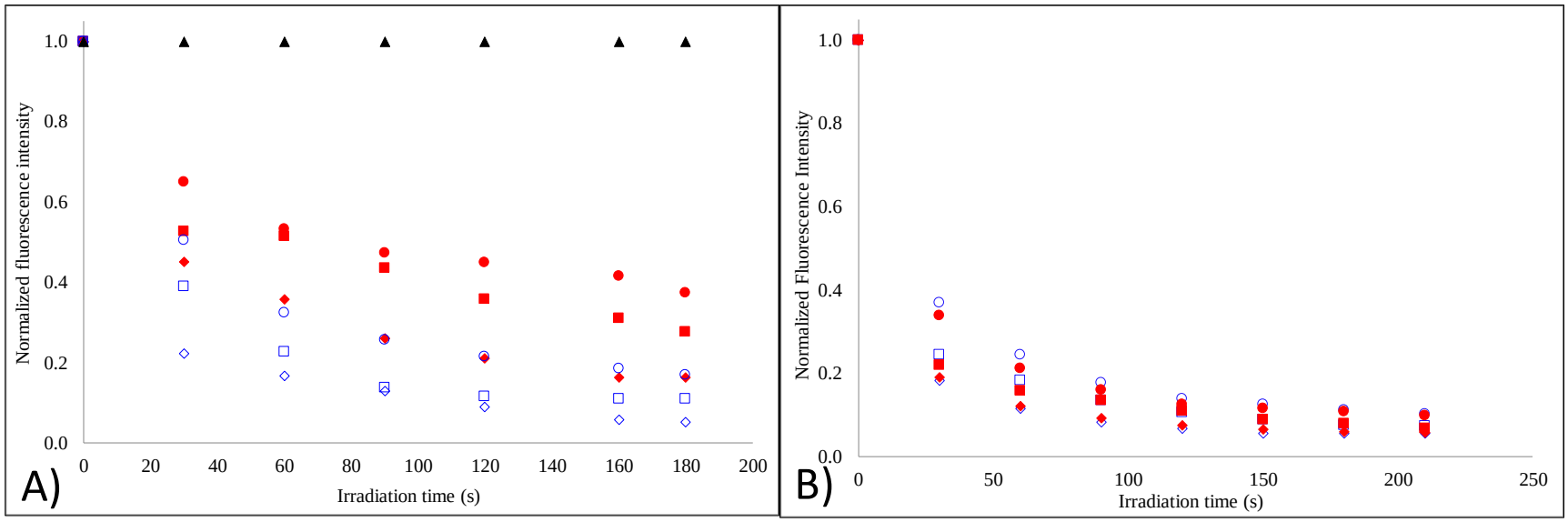


Figure 2. Normalized fluorescence intensity against irradiation time. A) NPs formulated *via* nanoprecipitation. Solid symbols: Run 3, open symbols: Run 4, Table 1. B) NPs formulated *via* E/E without (Open symbols) and with (Solid symbols) *in situ* CuAAC click chemistry (Runs 7 and 9, Table 1).

Irradiation power = 320 mw/cm² (○,●); 620 mw/cm² (□,■); 1150 mw/cm² (✧,♦); without irradiation (▲).

NPs core. Increasing $F_{PNBA,}$ and consequently NBA monomer units ratio, will produce higher concentration of carboxylic acid salts in PBS/H_2O phase. Consequently, the NR environment will turn faster more hydrophilic in case of copolymers with higher PNBA weight fraction, leading to a lower fluorescence intensity.

Secondly, NR-loaded PNBA-based NPs made by nanoprecipitation of Dex(15)-*g*-14$PNBA_{3500}$ (Run 3, Table 1) were chosen to study the effect of intermittent UV irradiation. Initial fluorescence intensity at λmax was measured, then such dispersion was irradiated for 30 s (320 mW/cm²). Fluorescence intensity was again measured just after, then the dispersion was left without irradiation for 30 s (Fluorescence intensity was measured every 10s). This operation was repeated several times. As shown in Figure 3, one can see an instantaneous decrease of NFI after each irradiation, leading to a stepped curve. During stirring periods, that means without UV irradiation, we observed a partial recovery of the fluorescence emission intensity that may be attributed to a balancing process of the NR between more or less hydrophobic parts of the disrupted NPs. These results confirm the high photosensitivity of PNBA-based NPs and their potential as light-triggered DDS. To the best of our knowledge, such discontinuous irradiation was never applied to PNBA-based DDS.

III.4) NR-loaded NPs made by E/E

NPs formulated by E/E were made carrying out or not an interfacial *in situ* CuAAC click chemistry (Runs 6-9, Table 1). Due to the very low quantity of NR loaded into such NPs, no significant NPs mean diameter variation was still observed.

Firstly, NPs were irradiated under various irradiation power and time and NFI evolutions were drawn (Figure 2B). As previously, the decrease of the NFI *versus* the irradiation time was depending on the irradiation power until reaching a plateau corresponding to the NR

fluorescence in PBS/H_2O phase, that also contains PAA or P(AA-co-NBA) (case of "unclicked" NPs), Dex-*g*-PAA or Dex-*g*-P(AA-co-NBA) (case of "clicked" NPs). More important, the presence of triazole rings produced during the interfacial *in situ* CuAAC click chemistry does not significantly affect the release of NR. Secondly, and according the no-influence of the triazole rings presence on the NR release, the effect of discontinuous UV irradiation on NR-loaded "clicked" NPs was studied (Figure 3). As explained before, NFI decreased after each irradiation, then increased while stirring due to the partially recover of NR fluorescence. These results are in concordance with those observed on NPs formulated by nanoprecipitation.

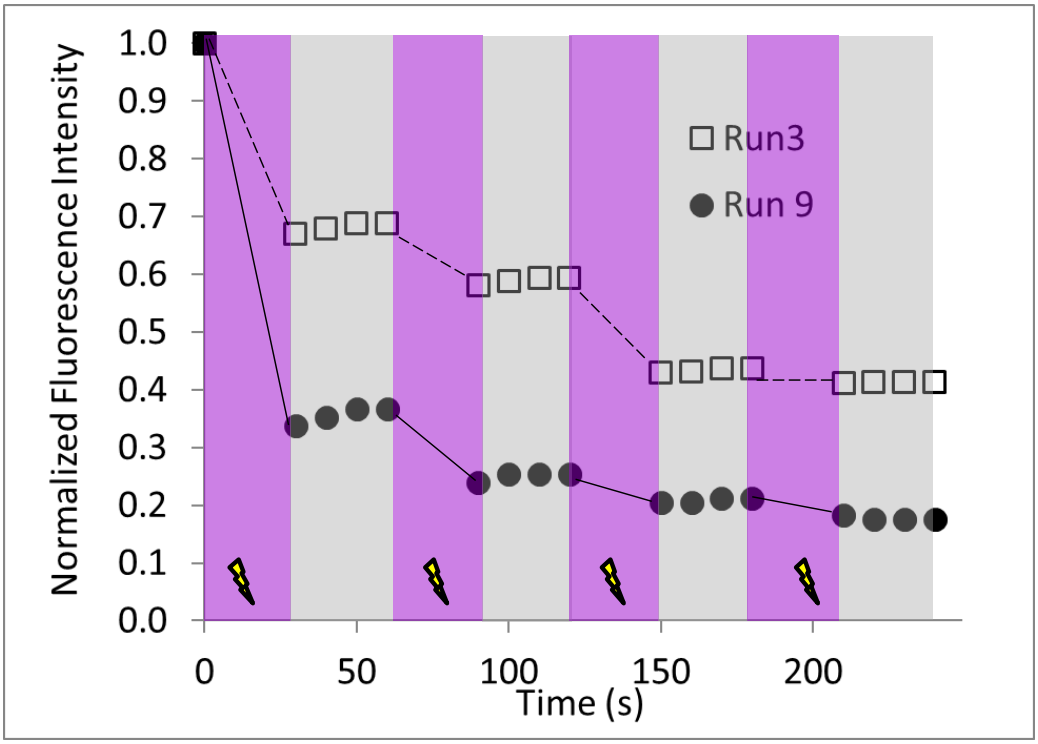


Figure 3. Normalized fluorescence intensity against intermittent irradiation (30 s irradiation, 30 s without irradiation). NPs formulated *via* nanoprecipitation (Run 3, Table 1) or *via* E/E (Run 9, Table 1). Irradiation power = 320 mw/cm².

In comparison with NPs made by nanoprecipitation, one can see that NFI decreasing was faster in case of NPs made by E/E due to the higher hydrophobicity (and thus photosensitivity) of the NPs core. A pure PNBA core is present in NPs made by this process, while NPs formulated by nanoprecipitation are made with a less hydrophobic core. The variation of the NR kinetics release is thus easily obtained by varying the chemical composition of the Dex-g-PNBA used within the nanoprecipitation process, while faster release is observed in the case of NPs made by E/E, whatever the occurrence of the interfacial *in situ* CuAAC click chemistry.

III.5) DOX-loaded NPs made by E/E

To investigate the potential use of PNBA-based NPs to light-triggered release a common drug, Doxorubicin (DOX) was arbitrary selected herein. DOX is easy obtained by neutralization of Doxorubicin hydrochloride (DOX, HCl) with an excess of triethylamine. DOX is a very famous anticancer drug used in chemotherapy treatment of several types of cancer (breast, ovarian, colorectal…) and already exists in many pharmaceutical forms (Adriblastin®, Caelys®, Myocet® or Doxil® for instance). DOX, HCl solubility in water (0.98 g/L) is divided by almost four after neutralization to DOX (solubility = 0.271 g/L).[45] Consequently, DOX is soluble in organic solvent as DCM used in the E/E process to formulate NPs.

After checking the stability of DOX during the sonication step within this process, 15 mg of DOX, HCl (that's mean 14 mg of DOX) was arbitrary blended with 25 mg of PNBA in the initial DCM phase (initial DOX weight fraction: 0.359) to formulate DOX-loaded "clicked" and "unclicked" PNBA-based NPs. As shown in Table 2, adding DOX into formulation increases the NPs mean diameter (109 *vs* 275 nm, Runs 1 and 3, for instance). This increase may be due to both higher weight concentration and molecular weight of PNBA used; but increasing

Table 2. Empty or DOX-loaded NPs formulated by E/E.

Run	Interfacial CuAAC	PNBA Feed	[b] DOX Feed	[c] Z-Average diameter (nm)	[c] PDI	[d] DL (%)	[d] EE (%)	[e] D (m^2/s)
1	Without	$PNBA_{7,900}$-Br	NO	109±2	0.144			
2	Without	$PNBA_{10,300}$-N_3 [a]	NO	128±2	0.090			
3	Without	$PNBA_{10,300}$-N_3	YES	275±2	0.117	33	88	$1.3\ 10^{-21}$
4	With	$PNBA_{10,300}$-N_3	NO	118±1	0.103			
5	With	$PNBA_{10,300}$-N_3	YES	243±2	0.127	29	73	$9.4\ 10^{-22}$

(a) 40 mg of PNBA were dissolved in 1 mL of DCM to formulate NPs.

(b) 15 mg of DOX, HCl were neutralized with 5 eq. of triethylamine, then dissolved in the PNBA/DCM solution to formulate NPs.

(c) Estimated by DLS.

(d) DOX loading (DL) and encapsulation efficiency (EE).

(e) DOX diffusion coefficient from PNBA environment to PBS/H_2O phase.

these parameters led to NPs with a Z-average diameter equal to 128 nm (Run 2, Table 2). Significant increases of the Z-average diameter after DOX loading were already reported by several authors for significant DOX loading (DL).[46,47] After formulation, DOX-loaded NPs were characterized to estimate the DOX loading (DL) and encapsulation efficiency (EE). Direct estimation of the loaded DOX was made by ^{1}H NMR after dissolving such loaded NPs in DMSO-d_6 (Figure S1), when indirect estimation was made by HPLC. Very similar DOX amounts were estimated by these two methods. As shown in Table 2, such DL values were not significantly influenced by the occurrence of the *in situ* interfacial CuAAC click chemistry as DL was almost 30% when EE varied from 73 to 88%.

DOX release was firstly investigated by diffusion at 37 °C. DOX-loaded "clicked" or "unclicked" NPs were dispersed in PBS medium, then left to diffuse for 2 days. Perfect sink conditions were checked by controlling that total diffusion of DOX will not exceed one-tenth of maximum solubility in such a medium (0.579 mg/mL as estimated by HPLC). Release curves were drawn (Figure 4A) and whatever the occurrence of the *in situ* interfacial CuAAC click chemistry, the release profiles were similar and almost 18% of the loaded DOX were released after 48 h diffusion. According to Siepmann's work, such diffusion profiles can be modeled during the first 40% fractional release using approximative equation (3) and considering PNBA-based NPs as spherical matrix systems and under the assumptions of perfect sink initial and boundary conditions, of not significantly swelling of the NPs and of a constant diffusion coefficient (D)[48]:

$$\frac{M_t}{M\infty} = 6\left(\frac{\mathrm{D}\times\mathrm{t}}{\pi\,\mathrm{R}^2}\right)^{1/2} - 3\,\frac{\mathrm{D}\times\mathrm{t}}{\mathrm{R}^2} \qquad (3)$$

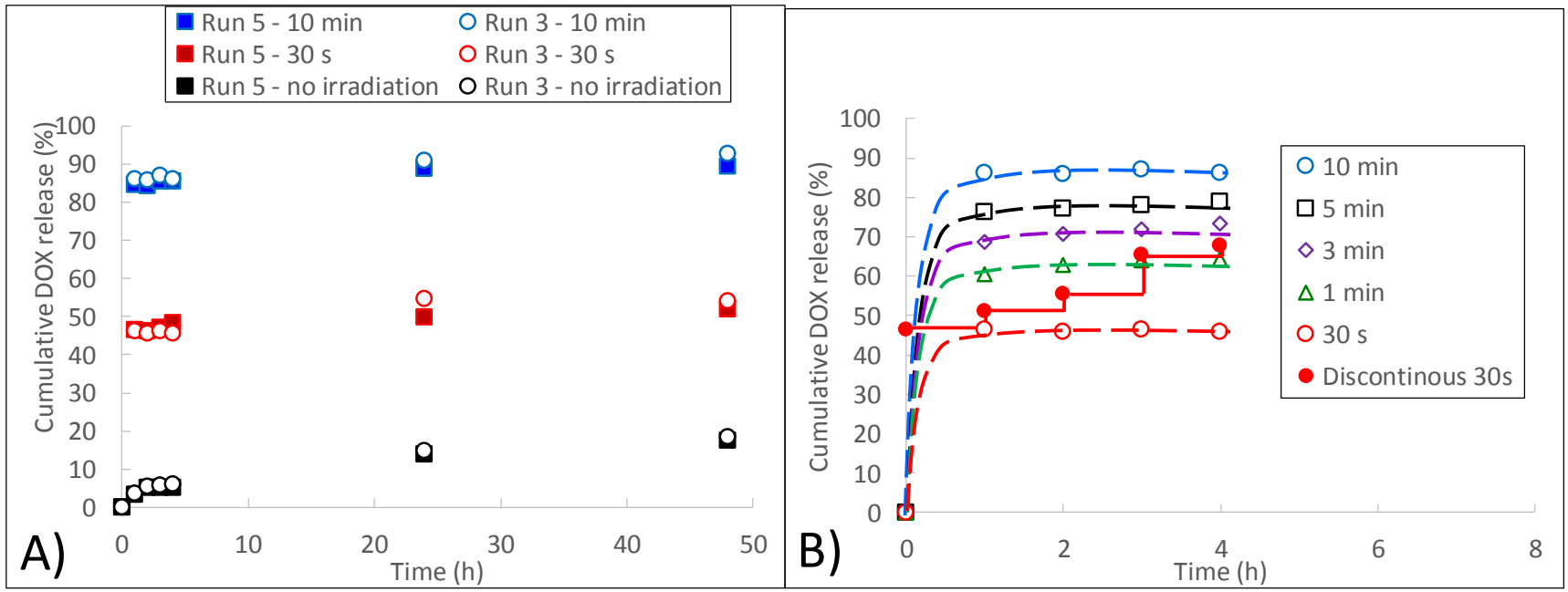


Figure 4. Cumulative DOX release from Dox-loaded PNBA-based NPs during 48 h. A) Release without irradiation, or due to 30 s or 10 min irradiation (Runs 3 and 5, Table 2). B) Release under 30 s discontinuous irradiation or 30 s to 10 minutes continuous irradiation (Runs 3, Table 2). Irradiation power: 60 mW/cm^2.

where M_t and M_∞ denote the cumulative amounts (g) of DOX released at time t and infinity, respectively. D is the diffusion coefficient of the DOX (m^2/s), t is the time (s) and R is the radius of the NPs.

Accordingly, the DOX diffusion coefficients (D) were estimated from "clicked" and "unclicked" PNBA-based NPs and values were between 9.4 10^{-22} and 1.3 10^{-21} m^2/s (Table 2). Considering similar mean diameters whatever the NPs (275 *vs* 243 nm), these D values seem similar (almost 10^{-21} m^2/s) and not significantly influenced by the occurrence of the *in situ* interfacial CuAAC click chemistry. Moreover, these D values are consistent with that previously reported in case of the DOX release in PBS medium out of PVA-covered crosslinked poly(2-hydroxyethyl methacrylate) (PHEMA) NPs (mean diameters almost 200 nm).[49] With such swelled PHEMA-based NPs, D were estimated between 2.5 to 4.5 10^{-21} m^2/s, that is almost thrice the value found in the present paper, in agreement with the higher hydrophobicity of PNBA *vs* PHEMA NPs cores. To the best of our knowledge, no other paper deals with the DOX diffusion from PNBA-based NPs. Nevertheless, we must mention one paper dealing with DOX diffusion through PNBM-core micelles (DL lower than 10%, average size almost 30 nm) but no estimation of the diffusion coefficient (D) was done.[37]

Secondly, the light-triggered DOX release was investigated. We could irradiate NPs using irradiation power equal 320 mw/cm² but this power led to a very important cytotoxicity of Caco-2 cells, while 100% Caco-2 cells viability was still observed after 48 h incubation by applying 30 s irradiation with a power of 60 mW/cm^2.[40] Consequently, PNBA-based NPs dispersions were irradiated using this low power for several times to observe progressive DOX release. The released DOX amount was estimated by HPLC just after the irradiation and during 48 h, leaving time for DOX to diffuse out the irradiated NPs (Figure 4A). Actually, the normalized scattered

light intensity of NPs dispersion decreased by almost 8% after 30 s irradiation upon such irradiation power; meaning a very slow NPs disruption.[40] As shown in the case of "unclicked" NPs (Run 3, Table 2), 30 s irradiation led to immediately release 44% of the loaded DOX. Then, a slow and progressive release of the DOX occurred through the irradiated NPs to reach 53% after 48 h, that is very high in comparison with the DOX diffusion out of native NPs. Such influence of the irradiation on the progressive release was observed whatever the irradiation time. For instance, after 10 min irradiation, the amount of DOX released is higher than 90% after 48 h (Figure 4A). The longer the irradiation time, the greater the amount of DOX released, in accordance with the photosensitive nature of the PANB core. Actually, we already reported the longer the irradiation time, the more noticeable disruption of PANB-based NPs.[40] Moreover, whatever "clicked" and "unclicked" NPs, the release profiles were very similar proving the non-influence of the *in situ* interfacial CuAAC click chemistry on the light-triggered release of the DOX (Figure 4A).

Finally, discontinuous UV irradiation was studied to module the DOX light-triggered release. "Unclicked" PNBA-based NPs were irradiated for 30 s using irradiation power 60 mW/cm², then the released DOX amount was estimated by HPLC just after the irradiation. Irradiated NPs dispersion was left stirring 1 h without irradiation, then next 30 s irradiation was done and released DOX amount was again estimated, and so on. As shown, an increase of the released DOX amount was observed after each 30 s irradiation leading to the stepped curve (Figure 4B). By this way, after 4 (30 s irradiation/1 h stirring) cycles, more than 67 % of loaded DOX were released, that is higher than the value observed with NPs irradiated during 30 s and left 4 h stirring. More precisely, an increase of almost 50% of the DOX released was attained when using discontinuous irradiation. This result highlights the possibility to step-improve the amount

of DOX released, while ensuring the no-cytotoxicity of the treatment using light-sensitive PNBA-bases NPs as 100% Caco-2 cells viability was still observed after same incubation time by applying 30 s irradiation.[40].

IV) CONCLUSION

Firstly, photo-sensitive PNBA-based NPs were formulated by two processes: i) nanoprecipitation of preformed Dex-*g*-PNBA without additional surfactant, ii) E/E carried out using alkynated dextran as surfactant and PNBA-N_3 as hydrophobic polymeric chains. By this later way, a physically adsorbed dextran shell covers the PNBA NPs core. But, carrying out an interfacial *in situ* CuAAC click chemistry, this dextran shell is covalently linked onto the PNBA core. "Clicked" and "unclicked" PNBA-based NPs were consequently formulated.

Secondly, NR was selected as hydrophobic fluorescent probe to investigate the loading of such NPs, then the release through the NPs. No NR release occurred by simple diffusion for two weeks but was observed due to the progressive disruption of such NPs under UV-irradiation, according to the photolysis of PNBA parts. Moreover, the release kinetics were not significantly influenced by the occurrence of the interfacial CuAAC click chemistry, while being influenced by the chemical composition/hydrophobicity of the photosensitive NPs core and the irradiation parameters (power, time, continuous or intermittent irradiations).

Finally, Doxorubicin was loaded into "clicked" or "unclicked" PNBA-based NPs. A DOX loading of about 30% was attained leading to an increase of the NPs mean diameter. A slow DOX diffusion from the NPs core to PBS aqueous phase was observed (18% after 48 h at 37 °C) allowing to estimate the DOX diffusion coefficient about 10^{-21} m^2/s. 30 s irradiation power 60

mW/cm^2, which proved to be no-cytotoxic towards Caco-2 cells, is enough to immediately release 44% of the loaded DOX, that increases to 53% after 48 h incubation. More interesting, such light-triggered DOX release can by modulated using a discontinuous UV irradiation as 67% of release was observed after 4 cycles (30 s irradiation/1 h stirring).

All these results demonstrated the potential applications of these Dex-covered PNBA-based NPs to control and modulate drugs release. In the very next future, DOX-loaded PNBA-based NPs will be investigated towards Caco-2 cells in cancer treatments.

V) SUPPLEMENTARY MATERIAL

DOX encapsulation, NFI of NR-loaded NPs without irradiation. HPLC calibration curves of DOX elution.

VI) ACKNOWLEDGMENT

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