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Molybdenum cluster loaded PLGA nanoparticles as efficient tools

against epithelial ovarian cancer

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ABSTRACT

In this study, poly (lactic-co-glycolic) acid nanoparticles loading inorganic molybdenum octahedral cluster were used for photodynamic therapy (PDT) of ovarian cancer.

Three compounds, ((C4H9)4N)2[{Mo6Br8}Br6], $Cs2[{Mo6Br8}Br6]$ cluster and Cs2[{Mo6I8}(OOC2F5)6] denoted TMB, CMB and CMIF were studied following their incorporation in nanoparticles by a nanoprecipitation method. All resulting nanoparticles exhibited physico-chemical characteristics such as size and zeta potential compatible with cellular uptake. All cluster compounds tested were shown to produce singlet oxygen in vitro once released from their nanoparticulate system. Confocal images showed an internalisation of cluster loaded nanoparticles (CNPs) in A2780 ovarian cancer cell line, more efficient with CMIF compared to CMB or TMB loaded nanoparticles. In vitro cellular viability studies conducted on A2780 cell line treated with non activated CNPs did not show any sign of toxicity for concentrations up to 15 µM. Following photo-activation, CNPs were able to generate singlet oxygen resulting in a decrease of the cellular viability, compared to non-activated conditions. Nevertheless, no significant differences between IC₅₀ with or without photo-activation were observed with TMB and CMB CNPs while for CMIF loaded nanoparticles, the photo-activation led to a significant decrease of cellular viability compared to the non activated condition and this decrease was independent of the P/C ratio. The strong photo-toxicity obtained for CMIF loaded nanoparticles with a P/C ratio of 2.5, as shown with half maximal inhibitory concentration (IC₅₀) value near 1.8 µM suggests that PLGA nanoparticles seem to be efficient delivery systems intended for tumor management and that CMIF can be further investigated as photosensitizer for PDT of ovarian cancer.

Key words: PLGA nanoparticle, molybdenum clusters, ovarian cancer cell line A2780, photodynamic therapy, singlet oxygen

1. INTRODUCTION

Ovarian cancer is the seventh most common cancer in women and the eighth most common cause of cancer death. Ovarian cancers are diagnosed at a late stage causing a poor prognostic (5-year relative survival rate of 29%) (Lheureux et al., 2019). There are several established risks and propective factors for ovarian cancer, most related to reproductive and hormonal factors (Webb and Jordan, 2017). Pathological diagnosis on tumour tissue is essential because ovarian cancer has different histological subtypes with different treatment approaches. Cytoreductive surgery is the first choice for the treatment, chemotherapy alone or combined with surgery is usually used in order to eradicate the non-removed foci. The value of cancer treatment is based on clinical benefit, toxicity, and improvements in patient symptoms or quality of life in the context of cost (Lheureux et al., 2019).

Photodynamic therapy (PDT) is a modern, innovative and non-invasive possibility of therapy. PDT is a highly selective method for the treatment of non-oncological diseases as well as cancers of various types and locations, clinically approved more than 30 years ago. PDT involves the selective uptake of a photosensitizer by neoplastic tissue, which is able to produce reactive oxygen species (ROS) upon irradiation with light, UV-VIS, near-infrared (NIR) or Xray (Kirakci et al., 2016; Kwiatkowski et al., 2018). Morevover, PDT offers the advantage to promote the generation of cytotoxic oxygen species only at the irradiated sites and a reduced toxicity to non-tumorous tissues.

ROS are produced as a result of metabolism of oxygen in living organisms, and play a significant role in cell signaling and homeostasis. Normally, ROS balance is maintained to prevent cell damages involving enzyme protection such as superoxide dismutase (SOD) or glutathione peroxidase (GPO) enzymes (Ochsner, 1997). However, increasing ROS level results oxidative stress when used as tumor suppressing agents in cancer treatment. ROS induce destruction of target cells and damage of tumor-associated vasculature and activate an

antitumor immune response, leading to tumor regression. The successful completion of this process is achieved by different mechanisms, including host immune responses and activation of cell death pathways such as apoptosis and necrosis (Kubiak et al., 2016).

Hexanuclear molybdenum cluster units with the general formula $[Mo_6L_8^iL_6^i]^{2-}$ (Figure 1) have been widely studied due to their photo-physical and photo-chemical properties (Jackson et al., 1996; Kirakci et al., 2012). In these clusters, inner ligands (Lⁱ) are halogen atoms covalently attached to Mo while terminal or apical ligands (L^a) are labile inorganic or organic ligands. When photo-activated, these complexes can either exhibit phosphorescence in the 550-900 window, of interest in bioimaging and biolabeling, and, in the presence of oxygen, they can act as powerful PS and generate singlet oxygen (Kirakci et al., 2012). The presence of oxygen in cancers cells can make them powerful PS destructing cells by oxidative stress (Nyman and Hynninen, 2004). As a result, hexanuclear metal atom clusters are potential candidates for theranostic applications (Ahmed et al., 2012; Krasilnikova et al., 2016; Solovieva et al., 2017, 2016). Three Mo₆ cluster based compounds were tested in our study, the salt $Cs_2[\{Mo_6Br_8\}Br_6]$ solid chemistry (CMB) prepared by state at high temperature, the salt $((C_4H_9)_4N)_2[\{Mo_6Br_8\}Br_6]$ (TMB) obtained easily in solution from the previous cluster by replacement of Cs^+ cations by tetrabutyl ammonium cations ((C_4H_9)₄N⁺) and $Cs_2[\{Mo_6I_8\}(OOC_2F_5)_6]$ (CMIF), prepared combining solid state and solution chemistries. To overcome the low stability of molybdenum cluster compounds in water at physiological pH that remains a barrier for biological applications, we used a nanoparticulate delivery system for these clusters.

TMB, CMB and CMIF clusters compound loaded nanoparticles (CNPs) may be useful for 2 other reasons: (i) photoluminescence properties of clusters is located in hypoxic regions enabling the detection of these foci and (ii) CNPs enable a passive targeting mainly to tumor

tissue and subsequently a reduced non-tumorous tissue accumulation and a further decrease in side effects.

The use of nanoparticle drug delivery systems is one approach to improve the physicochemical characteristics and the pharmacokinetic properties of many therapeutic agents. Most of these have been associated with limitations such as high toxicity, poor absorption rate, and a high rate of elimination thus limiting their bioavailability and subsequent pharmacological benefits, in particular for water insoluble drugs. Poly (lactic-co-glycolic acid) (PLGA) is Food and Drug Administration (FDA) approved biodegradable and biocompatible polyester polymer which have been widely used as a carrier for gene, vaccine, protein, and peptide delivery (Acharya and Sahoo, 2011; Danhier et al., 2012). A wide variety of hydrophobic drugs like curcumin, coumarin, and many others have been successfully encapsulated in PLGA as nanoformulations for use in therapeutic applications (Brandhonneur et al., 2018; Dollo et al., 2020; Makadia and Siegel, 2011).

In this study, the efficacy of PLGA nanoparticles embedding inorganic hexanuclear molybdenum clusters intended for PDT of epithelial ovarian cancer was investigated. Three Mo_6 cluster-based compounds differing by the nature of the counter cations, the inner ligands and the apical ligands were loaded in nanoparticles by solvent displacement method TMB, CMB and CMIF. RG503H as carboxylic acid terminated PLGA with a 50:50 lactic to glycolic ratio was used and several polymer-to-cluster (P/C) mass ratios were tested 1, 2.5 and 5. The characterization of the different CNPs were previously published (Dollo et al., 2020). Singlet oxygen measurements were performed on TMB, CMB and CMIF cluster compounds released from their nanoparticulate delivery system to assess singlet oxygen production. *In vitro* studies on A2780 ovarian cancer cell line evaluated the cellular toxicity after treatment with CNPs (photo-activated or not) at several concentrations (1 to 50 μ M), IC₅₀ were calculated.

2. MATERIALS AND METHODS

2.1. Chemicals

Different salt of molybdenum hexanuclear unit, TMB, CMB and CMIF were synthetized in our team (ISCR, CSM, Rennes, France) according to published procedures (Amela-Cortes et al., 2016; Kirakci et al., 2005). Acid terminated poly lactic-co-glycolic acid (PLGA) 50:50, MW 24000-38000 (Resomer[®] RG503H), polyoxyethylene sorbitan monoleate (Tween[®] 20), 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) and Dimethylsulfoxide (DMSO) were purchased from Sigma Aldrich (St Louis, MO, USA). Macrogol 400 (PEG 400) was purchased from Inresa (Bartenheim, France). Analytical reagent grade acetone, acetonitrile and absolute ethanol were obtained from VWR international S.A.S. (Fontenay-sous-bois, FR).

2.2. Nanoparticles formulation and characterization

Formulation of CNPs was described by Brandhonneur et al. (Brandhonneur et al., 2018). Several polymer-to-cluster (P/C) mass ratios were tested, i.e. 1, 2.5 and 5. Briefly, an organic solution of polymer RG503H (5 to 15 mg) and TMB, CMB and CMIF cluster compound (5 mg) in acetone (5 ml) was slowly added dropwise (1ml/min) to 0.5% w/v aqueous polysorbate 20 solution (15 ml) under magnetic stirring (500 rpm) at room temperature, instantaneously forming a bluish colloidal nanosuspension caused by the so-called ouzo effect. Reduced pressure by a rotary evaporator Laborata 4000 (Heidolph, Schwabach, Germany) allowed to remove acetone.

Nanoparticle suspensions were used for the measurements of particles size, zeta potential, singlet oxygen production and cellular studies.

2.3. Singlet oxygen measurements

Singlet oxygen (${}^{1}\Delta g O_{2}$) emission measurements were realized by irradiating the 3 CNPs suspensions (TMB, CMB and CMIF, with a P/C ratio of 2.5) with a laser diode ($\lambda_{exc} = 375$ nm), using a near infrared photomultiplier tube Hamamatsu H10330C-75 PMT as photon counting detector (Hamamatsu Photonics France, Massy, France). Measurements were performed with ethanol solutions obtained after the release of cluster compounds from CNPs at 1 hour (Dollo et al., 2020), immediately after preparation (D₀) and following 5 days of storage in the dark and under refrigeration (D₅).

2.4. Cellular studies

2.4.1. Cell culture

A2780 cell line (human ovarian cell carcinoma) was purchased from Sigma (Sigma Aldrich, France). A2780 cells were cultivated at 37° C 5% CO₂ in medium RPMI 1640 supplemented with 10 % fetal bovine serum and 1% (v/v) penicillin/streptomycin. Cells were cultured in 75 cm² Nunclon EasYFlask from (Thermo Fisher Scientific, Waltham, USA).

2.4.2. Phototoxicity activity and cellular viability

 10^3 cells/cm² were seeded in a flat bottom 24 well plate TPP® tissue culture plates (PP Techno Plastic Products AG, Switzerland) and incubated. At confluence, cell culture was removed and replaced with RPMI 1640 medium without serum. Cells were treated with different clusters (TMB, CMB and CMIF) loaded nanoparticles at several cluster concentration i.e. 1, 2, 5, 10, 15, 20, 25, 30, 40 and 50 μ M of cluster and incubated for 6 hours. Then, cluster compounds were stimulated at 365 nm for 10 min to release singlet oxygen using a one tube UV irradiation lamp with a power of 6W and an irradiance of 0.61 mW/cm². UV lamp was purchased

from Vilber Lourmat (Collégien, France). Afterwards, cells were re-incubated to complete 24 hours. Non-treated cells (negative control) were considered as 100% viability and 10% DMSO treated cells (positive control) were considered as 0% viability. The cellular viabilities were determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. MTT was added to cells at concentration of 2 mg/ml for 2 hours, then cell medium was aspirated and 200 µl DMSO were added to dissolve formazan crystals. 25 µl of formazan solution was transferred to flat bottom 96 well plate TPP® tissue culture plates (PP Techno Plastic Products AG, Switzerland) and was completed to 100 µl using DMSO was performed. Finally, cells viability was determined at 560 nm using POLARstar Optima microplate reader (BMG labtech, Ortenberg, Germany). Cell viability percents were normalized to non-treated cells by taking the positive control values into consideration. In order to differentiate the toxicity related to stimulated cluster form that related to cluster itself, control plates were made under the same experimental conditions excepting the step of cluster stimulation.

2.4.3. Confocal microscopy

Microscopy was performed using an Axio Observer 7 confocal microscope with an Airyscan detector (Zeiss, Oberkochen, Germany) with A2780 cells cultured under standard conditions on LabTek II culture chambers at 50.10³ cells/chamber. At confluence, cell culture was removed and replaced with RPMI 1640 medium without serum. Cells were treated with different clusters (TMB, CMB and CMIF) free and embedded in loaded nanoparticles at 15 μ M of cluster and incubated for 5 hours. Cells were washed by 2 x 400 μ L of PBS and incubated for 10 min at 37°C with 400 μ l of a wheat germ agglutinin-fluorescein conjugate (green) (WGA-FITC) solution in HBSS. Cells were washed 2 x 400 μ L of PBS and microscopy was performed. The clusters compounds were excited using a 405 nm laser (laser diode), and luminescence was measured using a 600-700 nm emission filter. Fluorescence of WGA-FITC was recorded at 520 nm using excitation at 488 nm (argon laser). The image reconstruction was performed with

the Zen software (Zeiss, Oberkochen, Germany) (Zeiss) and the analysis of colocalization with the ImageJ software.

2.5. Statistical analysis

Results are expressed as mean values \pm standard deviation (SD). A Mann-Whitney test was used for statistical analysis using GraphPad Prism 7.0 software, the level of significance was set at p < 0.05. IC₅₀ calculation were performed with R software (R foundation, Austria) with the "ic50" package.

3. RESULTS / DISCUSSION

TMB, CMB and CMIF exhibit photosensitization properties related to the release of reactive oxygen species after photo-activation (Kirakci et al., 2005). These activation by light is the main part of photodynamic therapy (PDT). To increase their aqueous solubility, to protect them from rapid degradation by hydrolysis and finally to increase their deposition in tumor region by the enhanced permeability, polymeric nanoparticles were developed as carrier systems with TMB, CMB and CMIF (Brandhonneur et al., 2018; Dollo et al., 2020).

3.1. Nanoparticles formulation and characterization

PLGA nanoparticles were prepared by the solvent displacement method also known as nanoprecipitation method (Fessi et al., 1989). In our study, several CNPs were obtained with 3 different clusters (TMB, CMB and CMIF) and 3 different polymers to cluster ratios (P/C = 1, 2.5 and 5). Nanoparticles size, polydispersity index (PDI), zeta potential and cluster loading were resumed in table 1. These physico-chemical parameters were extracted from our previous paper (Dollo et al., 2020). All nanoparticles size obtained were compatible with cellular uptake (between 76 nm and 145 nm). Indeed, sizes between 50 to 150 nm are needed for optimal uptake by tumor cells (He et al., 2010). According to previous reports on cellular uptake of polymeric nanoparticles, particle size is the most critical factor to consider for the cellular uptake of colloidal system as a lower size generally results in a better uptake by the tumor cells. PDI values were < 0.2 indicating a homogenous monodispersed profile of nanoparticles. Zeta potential measured values were all negative near to - 30 mV (Table 1). In fact, values higher than 30 mV (absolute values) correlate with good stability (Riddick, 1968). The negative zeta potential values are attributed to the charge given by PLGA polymer at the surface of nanoparticles. A direct correlation between zeta potential and cellular uptake was demonstrated (Khine et al., 2016).

3.2. Singlet oxygen

As already reported, the triplet states of $[Mo_6L^{i}_8L^{a}_6]^{2-}$ cluster units interact with molecular oxygen by energy transfer to form singlet oxygen singlet (${}^{1}\Delta g O_2$) (Jackson et al., 1996; Kirakci et al., 2013, 2012) and this physical process leads to a quenching of the cluster emission signal thus shortening its emission lifetime (Amela-Cortes et al., 2015; Efremova et al., 2016; Kirakci et al., 2012). In a previous paper we have shown that i) the emission of TMB embedded in PLGA matrix was quenched by molecular oxygen with subsequent production of singlet oxygen lowering cluster luminescence lifetime, ii) when clusters are entrapped within the polymer matrix, singlet oxygen emission was not observed meaning that they are somehow protected from oxygen and that cluster compounds must be released from the nanoparticles to generate singlet oxygen upon irradiation and iii) intrinsic properties of TMB cluster compound were not modified following their entrapment in PLGA nanoparticles (Brandhonneur et al., 2018).

Figure 2 presents ${}^{1}\Delta g O_{2}$ emission measurements performed on TMB, CMB and CMIF. As cluster compounds are more protected from oxygen when loaded in PLGA nanoparticles, we studied ${}^{1}\Delta g O_{2}$ production following cluster compound release from their corresponding PLGA nanosuspensions. Recently published data on cluster compound analytical stability when embedded in PLGA nanoparticles showed an analytical stability of 3 days for CMB, 7 days for TMB and one month for CMIF (Dollo et al., 2020). To ensure that the ability of ${}^{1}\Delta g O_{2}$ production is not modified when cluster loaded nanoparticles are used for *in vitro* studies 24 hours after their preparation, emission spectra from release medium (absolute ethanol) were recorded i) immediately after preparation (D₀) and ii) following 5 days storage in the dark under refrigeration (D₅). A weak ${}^{1}\Delta g O_{2}$ emission signal could be detected with CMB and TMB solutions following excitation at 375 nm. In these cases, an analytical treatment of the recorded signal (substraction of the signal corresponding to the tail of cluster emission) was needed to clearly observe the emission band with a maximum centered around 1270 nm corresponding to singlet oxygen. For CMB there was no difference in this emission during the 5 days period

while for TMB the signal was lowered. As already shown, the functionalization of the $\{Mo_6I_8\}^{4+}$ cluster core by $C_nF_{2n+1}COO^-$ groups leads an enhancement of optical properties compared to pure halides. It leads in particular to the highest quantum yield of singlet oxygen formation found for the $[Mo_6L_8^iL^a]^{2-}$ cluster units. Hence, upon excitation at 375 nm CMIF produce more ${}^1\Delta g O_2$ compared to CMB and TMB cluster based nanoparticles (Amela-Cortes et al., 2015; Khlifi et al., 2020; Kirakci et al., 2013, 2012). This is traduced by a more intense emission signal. No significant difference could be seen between D_0 and D_5 . From this experiment, we can conclude that all studied cluster compounds can still produce singlet oxygen after being released from their nanoparticulate system and that this production is not altered following several days of storage for CNPs containing TMB or CMIF.

3.3. Cellular uptake and in vitro cytotoxicity

Several studies indicate an action of photosensitizers on cells, bacteries and viruses (Beltrán et al., 2016; Berasaluce et al., 2020; Henke et al., 2016). Direct administration of cluster compounds is not pertinent in PDT owing to their poor aqueous solubility thus limiting their deposition on tumor. One of the possible approaches to overcome this drawback is the use of nano-objects. Nanoparticles have been engineered and applied for insoluble bioactive compounds by improving their aqueous solubility, their pharmacokinetics, their bioavailability and also by reducing their toxicity.

Nanoparticles prepared from natural or synthetic polymers are colloidal carriers with dimensions on the nano scale (10^{-9} m) particularly attractive to deliver a wide variety of therapeutic molecules for cancer treatment.

PLGA is one of the most used synthetic approved, biocompatible and biodegradable polymer to obtain nanoparticles drug delivery systems able to control drug release and to target cancer cells by passive targeting (due to their small size below 200 nm) or by active targeting (following nanoparticle surface functionalization with moieties such as folate, antibodies, peptides,...) (Kato et al., 2017).

Human ovarian cancer cells (A2780) were chosen for the biological evaluation. Uptake of cluster compounds and CNPs was monitored using laser scanning confocal microscopy. As illustrated in figure 3, solutions of cluster compounds in DMSO react differently depending on the nature of the counter cation. This emphasizes the influence of the latter on physico-chemical properties. The images obtained demonstrated the good penetration of CMIF solution into cells, on the other hand, TMB and CMB solutions adhered to cell membrane and did not penetrate into the intracellular space. However, when cluster compounds were formulated in particulate form, the intracellular uptake was visible for the 3 clusters loaded nanoparticles. These results showed an efficient internalisation of CMIF loaded nanoparticles compared to CMB or TMB loaded nanoparticles, exhibiting an uniform distribution of red fluorescence within the cytoplasm. PLGA nanoparticles have previously shown higher cellular internalization and higher toxicity with curcumin and kaempferol on cisplatin resistant ovarian cells line compared to free drugs (Luo et al., 2012; Yallapu et al., 2010). In a previous study, the stimulation of free TMB did not cause any cellular toxicity and the cellular viability was near 100% (Brandhonneur et al., 2018). These findings suggested the lack of cellular internalization for free clusters or their quick degradation by cellular enzymes, rendering them inappropriate for photo-activation. These results were validated by the confocal images.

The cellular viability on A2780 (ovarian cancer cell line) was determined following 24h incubation for 3 CNPs (TMB, CMB and CMIF) with 3 P/C ratios of 1, 2.5 and 5 (figures 4A, 4B and 4C for TMB; figures 4D, 4E and 4F for CMB; figures 4G, 4H and 4I for CMIF. The cellular viability was evaluated with CNPs with or without photo-activation, at relevant concentration ranging from 0.5 to 50 μ M. We evaluated the CNPs using MTT assay (figure 4). The yellow 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide is reduced by

metabolically active cells. The resulting intracellular uptake purple formazan can be solubilized and quantified by mean of spectrophotometry. The rates of metabolically active cells were determined against the negative control. Table 2 indicates the IC_{50} for TMB, CMB and CMIF loaded nanoparticles on A2780 cells obtained from cellular viability results with or without photo-activation.

The photodynamic toxicity was evaluated upon light irradiation (365 nm) after 6 h incubation time without serum to avoid interactions of the nanoparticles with serum proteins that are limiting the biological application (Hynek et al., 2018).

The results showed that non-activated TMB, CMB and CMIF loaded nanoparticles were not toxic up to 15 μ M (figure 4). These results were in adequation with the toxicity of the previously reported Mo₆ complexes (Kirakci et al., 2020). The cellular viability obtained for all nonactivated CNPs decreased with increasing cluster concentrations (for example, with CMIF loaded nanoparticles with a P/C ratio of 2.5, the cellular viability decreased from 98% to 2% when concentration was increaded from 0.5 to 50 µM). For all CNPs tested, there were no significant differences on cellular viability whatever the P/C ratio. However, we observed an important difference of cellular viability between these 3 clusters-based nanoparticles once photo-activated. TMB and CMB loaded nanoparticles showed no significant decrease in cellular viability upon stimulation at 365 nm compared to non activated conditions. For these TMB and CMB loaded nanoparticles, we did not observe significant differences between IC₅₀ without and with photo-activation. For example, IC₅₀ obtained with non activated TMB and CMB loaded nanoparticles with a P/C ratio of 2.5 were $18.22 \pm 0.22 \mu$ M and $17.88 \pm 0.38 \mu$ M, respectively while photo-activated nanoparticles led to IC₅₀ values of $9.59 \pm 0.40 \,\mu\text{M}$ and 13.63 \pm 0.62 μ M, respectively (table 2). However, for CMIF loaded nanoparticles, photoactivation led to a large decrease of cellular viability (figure 4). The activation of internalized clusters within cells initiates oxidative stress by liberation of reactive oxygen species such as singlet

oxygen, responsible for cellular death via interaction with cellular components and initiation of apoptosis signaling (Kessel, 2019; Kessel and Oleinick, 2018, 2010).

Referring back to control conditions, the cellular viability at 10 μ M significantly decreased from 98% to 9%, 80% to 2% and 86% to 5% with P/C ratios of 1, 2.5 and 5, respectively, showing that the effect of stimulated cluster was not increased when the P/C ratio was increased. We did not show any sign of toxicity for concentrations up to 10 μ M without photo-activation. A strong photo-toxicity with an IC₅₀ value of 1.81 ± 0.14 μ M was obtained for CMIF loaded nanoparticles with a P/C ratio of 2.5, indicating that these nanoparticles are the best photosensiting system. This IC₅₀ value was comparable to those obtained with ovarian cancer primary therapy. With cisplatin for example, an IC₅₀ of 1.5 ± 0.10 μ M was reported on A2780 cells (Altaf et al., 2019). However, our approach is based on intra-tumoral application with selective localisation, limiting adverse effects.

Conclusion

Three different Mo_6 based cluster compounds were successfully loaded in PLGA nanoparticles with 3 P/C ratios (1, 2.5 and 5). Nanoparticles exhibited homogenous profiles sizes and negative

zeta potential values compatible for cellular uptake. Cellular viability was evaluated on ovarian cancer cell line (A2780) with CNPs. At 10 μ M, no cellular toxicity was observed for 3 CNPs for all P/C ratios. CMIF loaded nanoparticules photo-activated at 365 nm led to a cellular toxicity comparable to non-activated conditions. IC₅₀ of CMIF loaded nanoparticles were significantly reduced. These results consider CMIF loaded nanoparticles as potential candidates for photodynamic therapy as the PLGA matrix can allow an increased intracellular uptake and can avoid cluster premature degradation. The next stage of this study will consist in evaluating i) ROS level inside A2780 cells induced by CMIF loaded nanoparticles and ii) secondary toxicity mechanisms as well as cellular photodestruction modes (apoptosis and necrosis).

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Figure captions

Figure 1: A. general structure of the hexanuclear molybdenum cluster unit $[Mo_6L^i_8L^a_6]^{2-}$ where Lⁱ is the inner ligand (Br or I in this study), L^a is the apical ligand (Br or (OOCC₂F₅)₆ in this study) and A+ is the counter cation ((C₄H₉)₄N+ or Cs+ in this study); B. schematic representation of Cs₂Mo₆I₈ (OOCC₂F₅)₆.

Figure 2: Emission spectra of singlet oxygen from each cluster compounds studied, TMB (A), CMB (B) and CMIF (C) following their release from nanoparticles immediately after preparation (D₀) and after 5 days storage refrigerated and protected from light (D₅). All spectra were recorded at λ exc = 375 nm; inset: analytical treatment of the same signal following baseline subtraction to enhance the singlet oxygen emission signal.

Figure 3: Localization of free TMB, CMB and CMIF (A, B and C) or cluster loaded nanoparticles (D, E and F) in A2780 cells. Confocal sections showing colocalization of free clusters or cluster loaded nanoparticles (red labeling) and cellular membrane with the WGA-FITX (green labeling).

Figure 4: A2780 cellular viability after 6 hours incubation with TMB, CMB and CMIF loaded nanoparticles, with P/C ratios of 1, 2.5 and 5 (A, B and C; D, E et F; G, H and I, respectively), followed by excitation at 365 nm. At confluence, cells were treated at 1, 2, 5, 10, 15, 20, 30, 40 and 50 μ M cluster concentration. MTT was performed at 24 hours. P express the significance level where * = P < 0.05, ** = P < 0.01 and *** = P < 0.005 respectively. Viability results are normalized to cells without treatment that are regarded as 100% viable Each bar represents the mean ± SD (n=3).

Figure 1



Figure 2



С

Figure 3

А







CMB







Figure 4



Table 1: TMB, CMB and CMIF loaded nanoparticles physico-chemical characteristics for different P/C ratios (1, 2.5 and 5). Each value is the mean \pm S.D. of 3 determinations from Dollo et al. 2020.

Cluster	Polymer to cluster ratio	Particle size (nm)	PDI	Zeta potential (mv)	Cluster Loading (%)
TMB - PLGA		85.7 ± 4.9	0.12 ± 0.02	-25.8 ± 4.4	50.9 ± 3.5
CMB - PLGA	1	75.7 ± 2.5	0.16 ± 0.02	-22.4 ± 9.8	73.9 ± 9.4
CMIF - PLGA		102.0 ± 2.6	0.16 ± 0.01	-38.2 ± 1.7	73.6 ± 12.9
TMB- PLGA		95.0 ± 6.1	0.12 ± 0.01	-42.2 ± 8.0	38.0 ± 13.5
CMB - PLGA	2.5	99.3 ± 4.6	0.12 ± 0.01	-47.8 ± 9.1	47.8 ± 12.0
CMIF - PLGA		124.7 ± 2.5	0.08 ± 0.02	-47.8 ± 4.6	69.8 ± 6.5
TMB - PL GA		1/17 + 6/1	0.09 ± 0.02	-24.8 ± 1.3	29.2 + 5.6
CMB - PLGA	5	141.7 ± 0.4 118.3 ± 4.3	0.07 ± 0.02 0.12 ± 0.01	-43.1 ± 6.5	34.9 + 3.0
CMIF - PLGA	5	144.7 ± 4.7	0.09 ± 0.03	-31.5 ± 3.6	55.0 ± 9.4

Table 2: IC₅₀ obtained with TMB, CMB and CMIF loaded nanoparticles for different P/C ratios (1, 2.5 and 5), not activated (IC₅₀) and photo-activated (IC₅₀ PA). Each value is the mean \pm S.D. of > 6 determinations.

Cluster	Polymer to cluster ratio	IC ₅₀ (μM)	IC ₅₀ PA (μM)
TMB- PI GA		24 11 + 0 46	18 18 + 0 33
CMB - PLGA	1	21.47 ± 0.42	10.10 ± 0.00 17.21 ± 0.45
CMIF - PLGA		24.18 ± 0.36	$3.66 \pm 0.09^*$
TMB - PLGA		18.22 ± 0.22	9.59 ± 0.40
CMB - PLGA	2.5	17.88 ± 0.38	13.63 ± 0.62
CMIF - PLGA		19.07 ± 0.14	$1.81 \pm 0.14*$
TMB - PLGA		10.68 + 0.10	12 10 1 0 22
CMB - PLGA	5	19.08 ± 0.19 17.72 ± 0.22	15.10 ± 0.32 14.52 ± 0.21
CMIF - PLGA	5	17.72 ± 0.32 23 21 ± 0 15	14.32 ± 0.31 2.38 ± 0.10*
		23.21 ± 0.13	2.50 ± 0.10

* p < 0.05

Graphical Abstract



CRediT author statement

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Declaration of interests

 \boxtimes The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

□The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: