Reviewer 1

Authors present the study of Radiotherapy Enhancement in Prostate Cancer Treatment by Titanate Nanotubes Engineered with Gold Nanoparticles and Docetaxel. They present the detailed description of the synthesis of complex nanoparticle however just few experiments are dedicated to its preclinical validation. The following attention shall be brought to authors’attention.

1.) Title: Since the presented experiments are mostly dedicated to process of synthesis (5 figures) of very complex nanoparticle and just 2 figures are dedicated to proper evaluation of the benefits of the treatment efficacy, I would suggest to modify the title of the article according its real achievements.

Answer: We thank the reviewer for his/her justified remark. For a better adequacy of the title with the contents of our manuscript, we modified the previous title for the following one: Titanate Nanotubes Engineered with Gold Nanoparticles and Docetaxel so as to Enhance Radiotherapy on Xenografted Prostate Tumors.

2.) Introduction: The introduction (line 49) mention epidemiology of prostate cancer in US but regarding the origin of authors and the journal, I would suggest to add the data regarding EU and/or world as well

Answer: Thank you so much for this interesting remark. A recent reference for the prostate cancer epidemiology in Europe (Ferlay, Colombet et al., Eur. J. Cancer 2018, 103, 356-87) has been added to the revised version of the manuscript.

3.) My biggest concern is related with part of introduction describing the benefits of nanoparticles to the standard cancer treatment. The authors describe the effect of biodistribution after i.v. injection, mechanism of EPR effect linked with i.v. injection (line 65), good biodistribution of Au-NPs after i.v. injection and its renal clearance that may be overcome with IoONts (121-127 line) etc., however in the article the authors use just i.t. injection. Thus, in my opinion, the introduction is not coherent with the experimental design and the achieved results.

Answer: We thank the reviewer for his/her remark. The introduction has been modified accordingly. our arguments for discussing intratumoral injection (IT) in contrast to systemic injection (IV) and the EPR effect were i) the efficiency of nanohybrids in IT compared to IV even with the EPR effect; ii) the maintaining of nanotubes on site thanks to the design of nanohybrids compared to circulating nanovectors; and iii) the very possibility of combined injection of nanohybrids with radioactive iodine grains during brachytherapy. A previous study not yet published showed a renal elimination of bare nanotubes within 24 hours, the results of which have strengthened us in the choice of intratumoral injection for the targeted objectives (the best possible activity within the tumor). As this part of the introduction was unclear, we have modified it and highlighted the changes in the manuscript.

4.) Results: In the results section authors describe that the nano-platform can be use for nuclear imaging and therapy. Which results support the possibility of imaging of TiONTs-AuNPs-PEG3000-DTX. If it is just a supposition than this statement should be presented with less strength.
Answer: We thank the reviewer for his/her remark, but we are not really sure to understand the question of the reviewer, but Figure 7 clearly shows that the fate of TiONts-AuNPs-PEG3000-DTX after intratumoral injection can be monitored by nuclear imaging. The main information from this nuclear imaging experiment lies in the fact that the hybrid nanoplatforms were retained in the tumor. Since the gold nanoparticles and indium ions are too small to be retained for a so much long time, we can deduce that the radiolabeled gold nanoparticles are bound to the titanate nanotubes. Effectively, we demonstrated in a parallel experiment (not yet published) that the radiolabeled gold nanoparticles escape from the tumor: 24 h after intratumoral injection, 90-95% of the injected dose were removed from the tumor when they are not grafted to large carriers. In the case of TiONts-AuNPs-PEG3000-DTX, approximately 70% of the injected dose were still present in the tumor.

The radioactivity is naturally assigned to the presence of Indium-111. If these radioisotopes were not complexed, they would diffuse outside the tumor. Moreover, the distribution of Au@DTDTPA gold nanoparticles radiolabeled with indium-111 is completely different (Figure R1 below). The absence of radioactivity outside the tumor (and in particular in the kidneys and bladder for radiolabeled gold nanoparticles or in the whole body for the free indium-111 ions, Figure R1 below) indicate that indium-111 ions and radiolabeled gold nanoparticles are not released (they are still grafted to the titanate nanotubes).

Fig. R1. Tomographic scintigraphy of the whole body of the rat ((a,c) sagittal projection and (b,d) posterior projection) acquired 24 h after intravenous injection of (a, b) Au@DTDTPA-111In nanoparticles and (c, d) 111InCl3 solution. The images were deliberately saturated in order to visualize the low activity zones. (from Nanoscale 2013, 5, 5930-5939)

5.) What is the structure and dimensions of final nanoparticle including DTX? Could you add TEM images?

Answer: We thank the reviewer for his/her remark. The dimensions of final nanohybrids are very similar to those of previous steps when standard deviations are considered. TEM observations did not provide conflicting information when final nanohybrids and initial ones are compared but the organic coating is transparent to the electron beam of TEM. Moreover, DLS measurements cannot be realized on our nanotubes (standard models are based on spheres not tubes) and other experimental models in DLS did not lead to realistic dimensions. The interesting information of hydrodynamic diameter/dimensions cannot be therefore provided for any of our nanotube-based nanohybrids. Additionally, general information/considerations are the following: bare nanotubes are ca. 170 nm in length and 10 nm in diameter (TEM determination); the APTES layer should add a few nanometers (1-5 nm) to the previous dimensions (based on DLS observation on spherical iron oxide
nanoparticles, J. Boudon et al. *Chem Commun.* 2013; the PEGylated layer should bring additional few nanometers (ca. 1-10 nm) to the nanohybrids (as observed on spherical iron oxide NPs in G. Thomas et al. *ACS Omega* 2019, 4, 2637-2648. https://doi.org/10.1021/acsomega.8b03283); gold nanoparticles surrounded by DTDTPA exhibit a hydrodynamic diameter of ca. 5.4 nm (Butterworth et al., *Nanomedicine* 2016, 11, 2035-2047. https://doi.org/10.2217/nnm-2016-0062); concerning DTX-PMPI, the maximum length in the extended configuration is above ca. 2 nm (Chemdraw measurement of minimized energy configuration). All these elements taken into account, the final nanohybrid (TiONts + the organic layer including AuNPs) should reach between 5 and 20 additional nanometers considering each constituting element end to end which should not represent the reality of our complex coating.

6.) Table 1. Explain the reproducibility column (n). Why the n of AuNPs-PEG3000-DTX is so low (2)?

**Answer:** At the moment the experiments were performed, we were limited in the rather expensive PMPI and DTX amount. In spite of that, the two different experiments realized led to the exact same results, hence the low n value.

7.) Figure 3. Include HAADF-STEM image of AuNPs-PEG3000-DTX or an explanation why it is not included.

**Answer:** Unfortunately, HAADF-STEM image including DTX was not included and cannot be: the high amount of organic coating around TiONts renders the image blurred and the resolution and focus were very difficult because of the sample degradation under the electron beam.

8.) Table 2. Include XPS analysis of AuNPs-PEG3000-DTX or an explanation why it is not included. 9.) Figure 4. Include data of AuNPs-PEG3000-DTX or an explanation why it is not included.

**Answer:** As mentioned in the manuscript, TiONts-AuNPs-PEG3000-DTX nanohybrids are relatively complex scaffolds including a very diverse organic layer and linkages, the attribution of peak components of which then becomes imprecise. Moreover, the relative amount of newly formed covalent bonds compared to the already existing ones is very low and below the limit of quantification of XPS ca. 1 atomic percent, as a consequence significant differences have not been observed because of this limitation of the technique. Therefore, XPS data of these TiONts-AuNPs-PEG3000-DTX nanohybrids were not included.

10.) Figure 5a. Include FTIR spectra of AuNPs-PEG3000-DTX or an explanation why it is not included.

**Answer:** As mentioned in the manuscript, TiONts-AuNPs-PEG3000-DTX nanohybrids are relatively complex scaffolds including a very diverse organic layer as well as various linkages, the attribution of vibration bands of which then becomes imprecise. Moreover, the relative amount of newly formed covalent bonds compared to the already existing ones is very low and the theoretical wave numbers unfortunately correspond already present bands possibly hiding the new ones. Therefore, FTIR data of these TiONts-AuNPs-PEG3000-DTX nanohybrids were not included.
11.) Figure 6. To prove the colloidal stability under physiological condition, the serum media should be examined.

Answer: Thank you so much for this interesting remark. When intravenous injection is considered, colloidal stability study in serum media would be an asset. However, in the case of intratumoral injection like in our study, colloidal stability is a little less important even if in our case, it has been nevertheless optimized and evaluated in phosphate buffered saline, since that is this medium that has been injected, as a physiological buffer, with our nanoparticles.

12.) Figure 7a. More prostate cell lines should be tested and the decrease of therapeutic activity of Docetaxel bound to nanoparticles should be discussed. For that the results of loading and release of the drug should be presented.

Answer: We thank the reviewer for his/her remark. First, another prostate cell line was also tested (22RV1 not shown) leading to almost the same observation, i.e. a decrease in IC50 in the case of DTX-bound nanoparticles and was not included eventually because no additional information was brought by this second assay. Second, in our study, concerning the design of our nanohybrids, we made the decision to have DTX covalently bound to nanotubes in order to be sure that the therapeutic molecule stands exactly in the same place than nanotubes. It means that the “loading” part corresponds to the grafting of DTX onto the nanohybrid coating at the end of PEG chains presenting thiol functions (the amount of which was determined by TGA). Then, there is no reason that the formed carbon-sulfur bond breaks afterwards in the in vivo environment. That is why a release profile of DTX is not relevant in our case, nanohybrid-bound DTX has been designed to interact while being attached accepting a decrease in the therapeutic activity of DTX although still cytotoxic.

13.) Figure 7b. The biodistribution of nanoparticles after i.v. injection should be explored.

Answer: This study has been performed only using intratumoral injection because we would like to develop these nanohybrids in association with iodine 125 seeds in brachytherapy for prostate cancer in case of clinical developments. During this procedure, radiation oncologists use specific needles to introduce iodine 125 seeds inside prostate gland and may use these same needles to inject nanoparticle at the end of the brachytherapy procedure. In these conditions it should not be relevant to evaluate the effect of intravenous injection of these nanohybrids. Since several preliminary in vitro studies showed cell internalization of our nanoparticles, intratumoral administration seems more relevant to target tumor cell than intravenous administration. Intravenous administration may lead to nonspecific targeting and may reduce tumor radiotherapy sensitization effect: the nanohybrid accumulation by EPR in tumor tissues will always be less than the amount in case of intratumoral injection. Moreover, as said previously, a previous study (not yet published) showed a renal elimination of bare nanotubes within 24 hours. The authors are therefore not convinced about the scientific interest of an intravenous biodistribution study in the present study. However, the relevance of intratumoral administration was initially not sufficiently argued and has been corrected in the manuscript.

14.) Figure 8. Regarding the current title of the article, this should be the most important figure of this manuscript, however very important controls are missing: i.) RT alone, ii.) AuNPs (IT) + RT, iii.) DTX (i.t.) alone. Also the curves reflecting the tumor growth should be included.
Answer: This study follows another study previously published which highlighted that TiONts-DTX+RT were more efficient than RT alone or than free-DTX + RT (Mirjolet et al., Int. J. Nanomed. 2017). In the present study the effect of grafted AuNPs has been evaluated on the nanohybrid TiONts-DTX previously studied. We agree with reviewer concerning the interest to evaluate the effect of AuNPs alone or associated with RT: one of our co-authors is an expert in AuNPs and has recently published a study on radiosensitization in prostate cancer (Butterworth et al. Nanomedicine, Future Medicine, 2016, 11 (16), pp.2035-2047. DOI: 10.2217/nnm-2016-0062). In this study, the authors concluded that PC3 tumor growth delay in animals treated with AuNPs-DTDTPA, show extended survival by 31% over animals receiving radiation only. However, the aim of our study was to improve the TiONts-DTX nanohybrids and not to compare them with free AuNPs. Moreover, as previously described with the same tumor model, AuNPs intra tumor injection alone without RT didn’t induce any effect on tumor volume (see Fig. below, extracted from Butterworth et al., Nanomedicine, 2016).

Interestingly, we highlighted close to a two-fold better efficacy when AuNPs have been grafted on TiONts-DTX and associated with RT (42% increase in tumor growth delay) compared to TiONts-DTX + RT without AuNPs (23% increase in tumor growth delay). However, the Au quantity injected (36.1 nmol AuNPs/animal either 7.5 µg AuNPs/animal and 15 µg Au@DTDTPA/animal) was significantly less than the quantity used in previous publication on AuNPs-DTDTPA alone (Butterworth et al. 2016, 160 µg of Au@DTDTPA/animal) thus showing the synergistic effect of the association of TiONts and AuNPs.

Concerning the presentation of our results, the tumor growth delay is a standard parameter commonly used to evaluate effect of ionizing radiation and to perform statistical analyses. However, if curves of all mouse for each treatment group are preferable, they can be found below and have been added to the supplementary information as well.
15.) Conclusions: The presented conclusion could not be stated based on the obtained results and should be completely reformulated.

**Answer:** Thank you for your remark. The conclusion was reformulated accordingly.

Line 554: not all grafting steps were precisely characterized. Data concerning TiONts-AuNPs-PEG3000-DTX are missing in several occasions (describe above)

**Answer:** The reviewer is correct, some characterizations have not been presented in the manuscript mainly because of relatively complex scaffolds of nanohybrids including a very diverse organic layer and various linkages, the attribution of peak components/bands of which then becomes imprecise. Significant differences have not been observed because the relative amount of newly formed covalent bonds compared to the already existing ones is very low and below limits of quantification. Therefore, inconclusive data of these TiONts-AuNPs-PEG3000-DTX nanohybrids were not included.

Line 560: Just the results of the stability in PBS are presented. In the physiological conditions the nanoparticles will be in the blood that contains serum, various proteins and other molecules not in PBS. The authors do not present the results confirming stability in vivo.

**Answer:** We thank the reviewer for his/her remark. A PBS suspension of nanohybrids has been injected directly into the tumor and not intravenously. Consequently, only the colloidal stability of nanohybrids in PBS was relevant to our study. After injection, we expected nanohybrids to be maintained into the tumor, if they become unstable at this stage, it is even better for the desired goal, limiting thus leakage to the rest of the organism.

Line 565: The results shows considerably less efficacy of the TiONts-AuNPs-PEG3000-DTX compared to free DTX thus it is not adequate to speak about potent therapeutic effect.

**Answer:** We thank the reviewer for his/her remark. Even if DTX alone has a greater cytotoxic activity than TiONts-AuNPs-PEG3000-DTX, the latter has still a satisfactory cytotoxic activity. Indeed nanohybrid-bound DTX has been designed to interact while being attached...
accepting a decrease in the therapeutic activity of DTX although still cytotoxic. Consequently, it is adequate because we have a better effect in the long term after intratumoral injection and radiation compared to DTX alone. Nanotubes allow immobilizing DTX into the tumor to improve the therapeutic effect. The term potent was probably too strong or equivocal, it has been replaced in the text by “long-term and increased therapeutic effect thanks to nanohybrid-maintained DTX into the tumor”.

Line 570: The comparison of efficacy in vitro of TiONts-AuNPs-PEG3000-DTX with TiONts-DTX is just theoretical and should not be stated as an conclusion without the experimental validation (cytotoxicity analysis provided in parallel single experiment)

Answer: In previous studies (Loiseau et al. Adv. Healthcare Mater. 2017, 6, 1700245. and Mirjolet et al. Int. J. Nanomed. 2017, 12, 6357), we realized in vitro tests of TiONts-DTX and we have described these results in this paper to compare them with TiONts-AuNPs-PEG3000-DTX. Consequently, we have added a cross-reference to these articles in the text to avoid suggesting that these are theoretical considerations.

Line 570: Further conclusion regarding internalization, without any experimental data, are just a supposition and should be experimentally proved.

Answer: The internalization process and kinetics of TiONts have been previously analyzed in vitro (Mirjolet et al., Radiother. Oncol. 2013). We have highlighted that TiONts were internalized by diffusion or endocytosis processes and stayed inside cells at least 10 days (MET analysis). As the point on internalization can be considered as a supposition, we cleared this part in the text and add a cross-reference to the mentioned article.

Line 575: If the TiONts-AuNPs-PEG3000-DTX platform is safe, why it was not contrasted with free DTX i.t. of injected i.v.?

Answer: We thank the reviewer for his/her question. This study has been performed only using intratumoral injection because we would like to develop these nanohybrids in association with iodine 125 seeds in brachytherapy for prostate cancer in case of clinical developments. During this procedure, radiation oncologists use specific needles to introduce iodine 125 seeds inside prostate gland and may use these same needles to inject nanoparticle at the end of the brachytherapy procedure. In these conditions it should not be relevant to evaluate the effect of intravenous injection of these nanohybrids.

Line 577: The authors state that the efficacy of therapeutic agent (DTX) is improved without even use the free drug in the experiment investigating the efficacy.

Answer: We thank the reviewer for his/her remark. We understand this lack of information. This publication is directly related with our previous paper (Mirjolet et al., Int. J. Nanomed. 2017) which has highlighted this effect. In these conditions it was not ethical to use additional animals to perform the same experiments that have been previously published. You can see below results previously demonstrated.
Finally, the authors would like to thank the reviewer for his/her valuable questions and comments definitively bringing our manuscript to a finer level of understanding and better emphasis of our results.

**Figure 4** Therapeutic effect of vehicle, free DTX, free TiONts, and TiONts-DTX associated or not with RT administered with three daily fractions of 4 Gy, 24 h after injection into PC-3 xenografted tumors.

**Notes:** Data are mean values of tumor volumes ± SD (n=7 per treatment group, eight treatment groups). P<0.013 (TiONts-DTX + RT vs DTX + RT or TiONts + RT), comparison performed using nonparametric Mann-Whitney test.

**Abbreviations:** DTX, docetaxel; RT, radiotherapy; TiONts, titanate nanotubes.