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Title: Understanding the mechanisms of cell death in photothermal nanomedicines

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- Nanomedicines can improve the delivery of photothermal therapy
- Changes in the intracellular environment will influence the type of cell death
- Combined gold and iron oxide can improve photothermal therapy

Dear Editor,

We have reviewed the recently published article by Movahedi *et al*. [1] and wish to make further comments and suggestions, on the research that has been presented in this publication. This study describes the characterisation of gold-coated iron oxide core-shell nanoparticles (NP) and the authors suitably justify the use of iron oxide and gold as materials, for efficiently delivering photothermal therapy (PTT) [2]. In addition to the cytotoxic effects elicited by light irradiating and therefore activating the NP, the authors also suggest and show the combination of NP-mediated PTT with radiotherapy to synergistically enhance treatment, termed as photo-thermo-radiotherapy. The concept behind this approach in combined treatment is to use nanomedicines to improve toxicity in cancer cells, whilst simultaneously reducing adverse toxicity to healthy tissue [3]. The KB human nasopharyngeal cancer cell line was used to model cancers and the human gingival fibroblast (HGF) cell line to model healthy tissue.

Our first criticism of this study is the characterisation of NP. A recent study by Mülhopt *et al.* highlights the challenges of minimising batch-to-batch variability of NP and their properties [4]. Factors such as particle size, purity and morphology can influence the overall effectiveness of NP batch-to-batch, which was not further investigated in this study. Plus, the long-term storage and stability of NP designed for potential clinical translation is crucial [5], which was also not commented on in this study.

The authors go on to describe the toxicity of NP in the two cell lines and show that a significant reduction in cell viability was only observed in the cancer cell line. No significant toxicity was observed in the fibroblast cell line. Two concerns are raised from this data. Firstly, it seems the NP alone, without light irradiation, induced

cytotoxicity in the cancer cell line. To some degree, this defeats the purpose of phototherapy, where light is required to activate to the photosensitiser. In the absence of light, the photosensitiser should remain stable and relatively non-toxic [6]. Secondly, the authors do not elaborate on why cytotoxicity was only observed in the cancer cell line and not the fibroblast cell line. No comment is made giving reason as to why this phenomenon was observed. Interestingly, the authors look at the uptake of NP, by measuring the amount of intracellular gold. However, this is only done on the KB cancer cell line and ideally should have also been done on the HGF cell line. This vital piece of data would have given credit to their results in Figure 2, and support their claim in that the NP are truly non-cytotoxic in HGF.

The final criticism of this study is uncertainty in the type of cell death, following combined photo-thermo-radiotherapy. The authors describe cell death by observing “disruption of plasma membrane, chromatin condensation, damaged mitochondria and swelling of nucleus membrane”. These events suggest necrosis as the mode of cell death [7], however, the authors argue that apoptosis is the mode of cell death due to the altered expression of the apoptosis regulating factors, Bax and Bcl2. We agree with the authors that apoptosis may have possibly been the mode of cell death, as it has previously been suggested that reaching 41-47°C during thermal ablation results in apoptosis and over 50°C can result in necrosis and an instant cell death [8]. We suggest further investigation into the mode of cell death to confirm apoptosis, through Annexin-V staining of cells and assays to determine caspase activity and the release of cytochrome c.

In summary, this paper highlights an advancement in PTT based nanomedicines and the advantages in combining PTT and radiotherapy to improve the outcomes of treatment. However, for future clinical translation, additional work addressing the concerns we have raised is essential.

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