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## Protein kinase C δ inhibitor peptide formulation using gold nanoparticles

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Dr. Wing Fu Lai

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Dear Dr. Lai,

We are submitting the revised manuscript entitled "***Protein kinase C δ inhibitor delivery using gold nanoparticles*** " for consideration as a publication in your Journal. We have revised the manuscript based on all comments from the reviewers and from the editorial office. A line-by-line responses are attached for your reference.

We appreciate your review of this work and are looking forward to hearing from you.

Sincerely,

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**TITLE:**

# Protein Kinase C-delta Inhibitor Peptide Formulation Using Gold Nanoparticles

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**KEYWORDS:**

Biomaterials, intravenous drug delivery, peptide therapeutics, drug delivery vehicle, amino acid, drug formulation

**SUMMARY:**

We have previously used a gold nanoparticle peptide hybrid to intravenously deliver a synthetic peptide, protein kinase C-delta inhibitor, which reduced ischemia-reperfusion-induced acute lung injury. Here we show the detailed protocol of the drug formulation. Other intracellular peptides can be formulated similarly.

**ABSTRACT:**

Protein kinase C-delta inhibitor (PKCδi) is a promising drug to prevent ischemia-reperfusion-induced organ injury. It is usually conjugated to a cell-penetrating peptide, TAT, for intracellular delivery. However, TAT has shown non-specific biological activities. Gold nanoparticles (GNPs) can be used as drug delivery carriers without recognized toxicity. Therefore, we have used a GNP/peptide hybrid to deliver PKCδi. Two short peptides (P2: CAAAAE and P4: CAAAAW), at a 95:5 ratio, were used to modify the surface properties of GNP. GNPs conjugated with PKCδi (GNP/PKCi) are stable in distilled water, 0.9% NaCl, and phosphate-buffered saline containing bovine serum albumin or fetal bovine serum. Intravenous injection of GNP-PKCi was previously

shown to prevent ischemia-reperfusion injury of the lung. This article outlines a protocol to formulate GNP/PKCi and assess the physiochemical properties of GNP/PKCi. We have used similar methods to formulate other peptide-based drugs with GNP. This article will hopefully draw more attention to this novel intracellular drug delivery technology and its applications *in vivo*.

**INTRODUCTION:**

Lung transplantation saves patients with end-stage lung disease[1]. However, serious complications after lung transplantation remain an obstacle. In the early stages following lung transplantation, primary graft dysfunction is the most harmful complication[1], and its primary cause is ischemia-reperfusion (IR)-induced acute lung injury[2].

Under cold preservation, metabolism in a donor lung is restricted to a very low level. However, reactive oxygen species and nitric oxide synthesis are activated due to the cessation of blood flow[3]. After transplantation, blood circulation is restored, and reactive oxygen species and nitric oxide generated during cold ischemia enhance inflammation and cell death, resulting in tissue injury.

To prevent IR injury, a protein kinase Cδ inhibitor (PKCδi) has been used in the heart, brain and lung[4-8]. These studies showed that PKCδi decreased inflammation and apoptosis during reperfusion. It has also prevented pulmonary IR injury in rats and in a lung transplant model[6]. PKCδi is usually conjugated with a cell-penetrating peptide, TAT, for intracellular delivery. However, it has been shown that the TAT peptide alone has non-specific biological effects, including promotion of angiogenesis, apoptosis, and inhibition of multiple cytokines[9-11]. Nanoparticles, small particles ranging from 1 to 100 nm in diameter[12], have been explored as candidates in facilitating drug delivery[13]. In particular, gold nanoparticles (GNPs) are regarded as noninvasive and nontoxic. Therefore, we have developed GNPs as drug delivery carriers for peptide-based drugs[14,15].

The surface of GNPs can be manipulated for specific applications such as molecular recognition[16,17], chemical sensing[18], imaging[19], and drug delivery. A GNP/peptide hybrid system has been developed, containing 20 nm GNPs and two short peptides (P2: CAAAAE and P4: CAAAAW) at a 95:5 ratio, to modify the surface properties of GNPs. The P2 peptide, with the negatively charged glutamic acid (E) at the end, stabilizes GNPs in an aqueous solution, and the P4 peptide, with the hydrophobic tryptophan (W) at the end, helps GNPs entrance into cells[14]. The cysteine (C) residue at the N terminus of these peptides contains a thiol group that can conjugate to the gold surfaces[14]. This peptide/GNP hybrid was further used to deliver PKCδi (CSFNSYELGSL). The optimized molar ratio of P2:P4 to PKCδi is 47.5:2.5:50. GNPs conjugated with PKCδi (GNP/PKCi) are stable in distilled water, 0.9% NaCl, and phosphate-buffered saline (PBS) containing bovine albumin or fetal bovine serum[14]. Intravenous injection of GNP/PKCi has been shown to prevent ischemia-reperfusion injury of the lung[15]. This article outlines a method to formulate GNP/PKCi and describes how to evaluate the physicochemical properties of GNP/PKCi. We have used similar methods to formulate other peptide-based drugs conjugated to GNP[20-22].

We hope this article will draw more attention to this novel formulation for intracellular drug delivery.

**PROTOCOL:**

**1. Preparation of Peptide Solutions**

1.1) Retrieve the peptides (P2: CAAAAE, P4: CAAAAW, PKCδi: CSFNSYELGSL) from the -20 °C freezer and thaw at room temperature (RT).

NOTE: Keep the bottle closed to prevent moisture from condensing on the peptides.

1.2) Weigh 0.01 g of each peptide on a microscale. Put each peptide into a separate 50 mL conical tube.

1.3) Add 18.74 mL of deionized (DI) water to the P2 tube.

1.4) Add 16.93 mL of DI water to the P4 tube.

1.5) Add 8.21 mL of 50% acetonitrile diluted in DI water to the PKCδi tube.

1.6) Vortex the peptide solutions briefly. Put the 50 mL conical tubes in a sonicator (40 MHz) for 5 min.

1.7) Bring the peptide solutions to a biosafety cabinet. All peptide solutions prepared should be 1 mM.

1.8) Transfer 1 mL of each peptide solution to a new 50 mL conical tube. Add 19 mL of DI water to the tubes of P2 and P4 and add 19 mL of 50% acetonitrile to the PKCδi tube, such that each solution is diluted to 50 µM and stored in its own tube.

1.9) Aliquot 1 mL of each 50 µM peptide solution into 1.5 mL microtubes.

1.10) Place all aliquots in a -80 °C freezer.

NOTE: Stock solution should be remade each month.

**2. Formulation of GNP/PKCi**

2.1) Remove the peptide solutions from the -80 °C freezer. Thaw them at RT. Bring them to a biosafety cabinet.

2.2) Add 475 µL of P2, 25 µL of P4, and 500 µL of PKCδi solution to 9 mL of 20 nm GNP solution ($7.0 \times 10^{11}$ particle/mL) in a 15 mL tube.

2.3) Exit the biosafety cabinet. Wrap the 15 mL tube with aluminum foil. Leave it on a shaker at RT overnight.

2.4) Return the samples to the biosafety cabinet. Aliquot 1 mL of GNP/PKCi into each 1.5 mL microtubes.

2.5) Centrifuge the tubes in a micro-centrifuge for 30 min at 15,294 x g at 4 °C.

2.6) Remove the supernatant from each tube under a biosafety cabinet.

NOTE: Be careful to remove the supernatant while ensuring that the GNP pellet remains intact and is not aspirated.

2.7) Re-suspend the pellet in the desired solvent according to the concentration required. Applicable solvents can be DI water, PBS, and 0.9% NaCl.

NOTE: Starting from 1 mL of GNP/PKCi, the GNP pellet contains $6.3 \times 10^{11}$ particles, based on the GNP concentration provided by the manufacturer. To administer $1.3 \times 10^{12}$ particles in 500 µL of 0.9% NaCl, we add 232 µL of 0.9% NaCl to each of three pellets. After pooling them together, we can then collect 500 µL of GNP/PKCi solution.

NOTE: Mix the desired solvent well before diluting the GNP/PKCi pellet, otherwise the GNP/PKCi will aggregate.

**3. Assessment of GNP/PKCi Hybrid Solubility**

3.1) Pour 0.5 mL of GNP/PKCi solution into an acryl cuvette. Place the acryl cuvette on a UV-Vis spectrophotometer and test the peak absorption[15].

**REPRESENTATIVE RESULTS:**

Care should be taken to evaluate the biophysical properties of the GNP/PKCi hybrid, as GNP tends to aggregate in a solvent. When GNP is aggregated, the color of the solution changes from pink to purple (**Figure 1A**). The UV-Vis spectrophotometer is able to detect changes more sensitively. If the GNP/PKCi is not aggregated, the peak of absorption should be at 525 nm (**Figure 1B**). If the GNP is aggregated, the peak of absorption will be shifted to the right. As an alternate method of analysis, when aggregates have formed, Δoptical density (ΔOD = OD at 525 nm - OD at 440 nm) decreases (**Figure 1C**).

**Figure Legends:**

**Figure 1: Quality of GNP/PKCi.** (**A**) Properly prepared GNP/PKCi is pink in color (left). Aggregated GNP/PKCi appears light purple (right). (**B**) Good GNP/PKCi preparation is stable in water, PBS, or 0.9% NaCl solution. Readings on a UV-Vis spectrometer indicated that the peak of absorption was

at 525 nm in all solutions. (C) An example of good and bad GNP/PKCi preparations. When the GNP was aggregated, the peak of absorption was shifted to the right. Moreover, ΔOD decreased.

**DISCUSSION:**

To ensure proper formulation, it is crucial that the PKCδi solution undergoes the sonication step outlined in step 1.6 of the protocol. The PKCδi peptide sequence contains hydrophobic moieties, so a sonicator assists in dissolving PKCi in the 50% acetonitrile solution. In addition, it is very important to mix the solvent meticulously, as outlined in step 2.7. The GNP/PKCi hybrid will not be well-formulated if these steps are not done properly due to aggregation of the PKCδi peptide[23].

GNP-based drug formulation provides several advantages. First, GNPs can be easily synthesized in well-controlled sizes, ranging from a few nanometers to ~100 nm[24]. Usually, smaller GNPs can deliver drugs into cells more efficiently than larger ones, since they can more easily diffuse into their target regions[25]. Second, GNPs are non-toxic *in vitro* and *in vivo*[26], rendering them safe drug carriers. Third, hydrophobic drugs can be loaded onto the modified GNPs[27]. Fourth, the surface chemistry of GNPs is readily modified for specific applications. In our studies, two short peptides were used to modify the GNP surface[14], stabilize them in physiological conditions, and impart new bioactivities[15]. The peptides were thoughtfully designed, with three regions including gold binding, spacing, and functional regions[14]. Specifically, the N terminus of the peptide has a cysteine (C) residue containing a thiol group that can bind with gold. The middle portion has four hydrophobic alanine residues to promote peptide assembly into a densely packed monolayer on the GNPs. The amino acid at the C terminus is a functional amino acid pointing outward, which can be used to manipulate the surface properties of the GNPs. The 95:5 ratio of these two peptides was systemically selected in a previous study[14]. The ratio between PKCi and P2/P4 peptides was also systemically tested and selected[15].

The GNP drug delivery system does have its limitations. GNPs are not cell type- or tissue-specific. GNPs are mainly accumulated in the lung, liver, and spleen after intravenous administration[28-30]. So far, this formula has only been tested in cell cultures and small animal models[15]. For translation to clinical applications, further studies on larger animal models are needed, and the system's pharmacokinetics, tissue distribution, and potential toxicity must be determined.

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**DISCLOSURES:**

The authors have nothing to disclose on this project.

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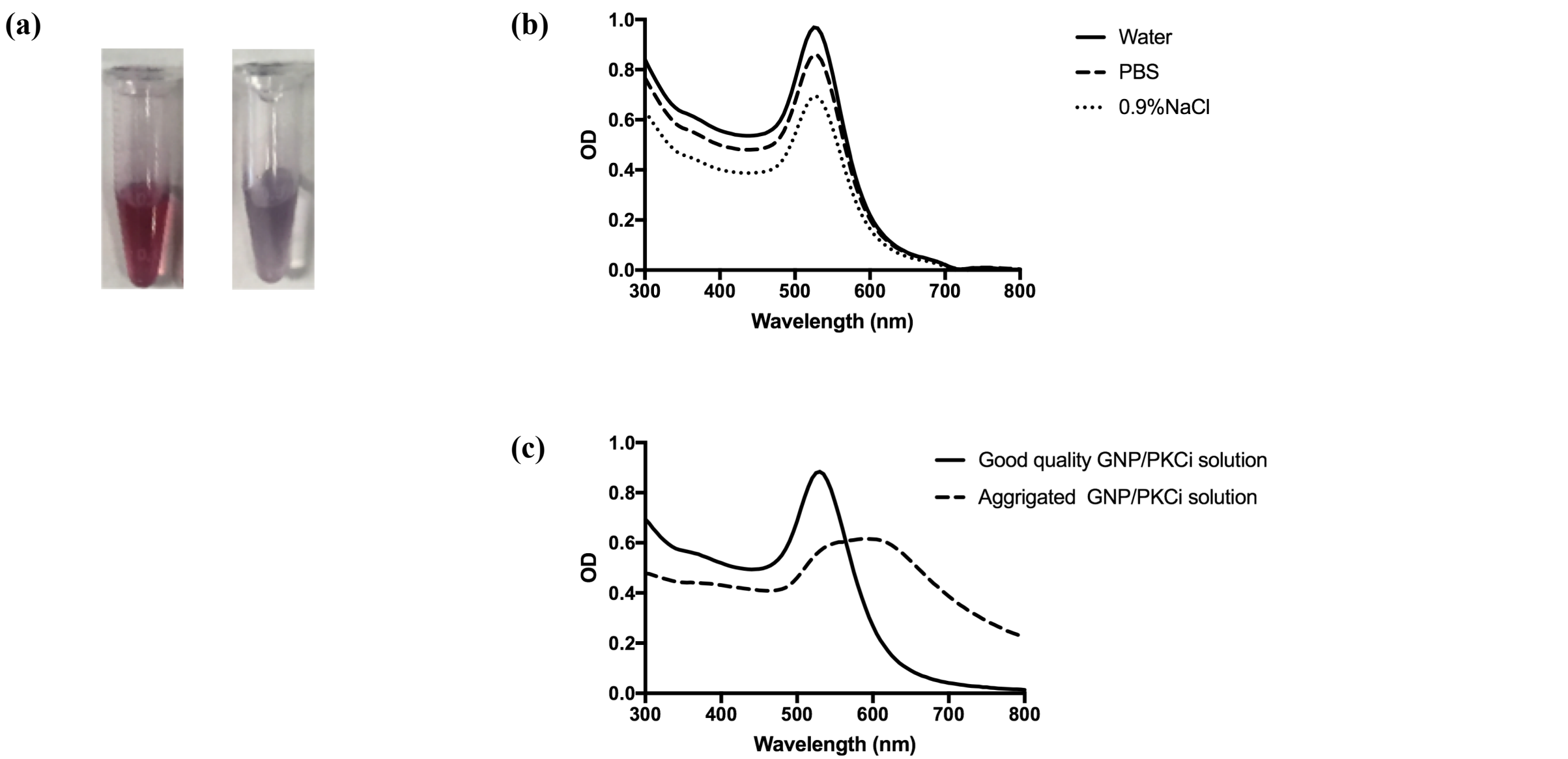
# Figure.1



| Name of Material/ Equipment | Company | Catalog Number | Comments/Description |
|---|---|---|---|
| negatively charged glutamic acid peptide (P2) | CanPeptide | | Sequence: CAAAAE-NH2<br>Length: 6aa<br>Modification: C-terminal amidation<br>Quantity: 50mg<br>Purity: >95% |
| hydrophobic tryptophan peptide (P4) | CanPeptide | | Sequence: CAAAAW-NH2<br>Length: 6aa<br>Modification: C-terminal amidation<br>Quantity: 50mg<br>Purity: >95% |
| δPKCi peptide | CanPeptide | | Seqeuence: CSFNSYELGSL-NH2<br>Length: 11aa<br>Modification: C-terminal amidation<br>Quantity: 50mg<br>Purity: >95% |
| Conical tube(50ml) | Corning Life | 3582070 | |
| Conical tube(15ml) | Corning Life | 3582096 | |
| Acetonitrile | Sigma-Aldrich | 271004-100ML | |
| Sonicator | Branson Ultrasonics Corp. | Branson 2510MTH | |
| Microtube | Diamed.ca | AD 150-N | |
| Gold nanoparticle solution | Ted Pella | 15705-5 | A particle size is 20nm |
| Rocking Platform shaker | VWR international | 40000-304 | |

| | | |
|---|---|---|
| Microcentrifuge | Eppendorf | 5417R |
| Acryl cuvette | SARSREDT | 67.758 |
| UV-Vis spectrophotometer | Agilent | Caty 60 UV-Vis |

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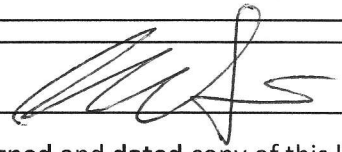
| Signature: |  |
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**Editorial comments:**

1. Line 2: Drug delivery has not been shown or demonstrated in this article. Please edit the title to best represent the protocol and results you are showing here.

Response: The title has been changed from “delivery” to “formulation” to represent our protocol and results.

2. Line 79,183,188,190 and 202: References?

Response: I added references #14, #24, #14 and 15, #14 and #15 on line 79, 183, 188, 190, and 202 respectively.


14 Yang, H., Fung, S. Y. & Liu, M. Programming the cellular uptake of physiologically stable peptide-gold nanoparticle hybrids with single amino acids. *Angewandte Chemie International Edition.* **50** (41), 9643-9646, doi:10.1002/anie.201102911, (2011).

15 Lee, D. *et al.* Effective delivery of a rationally designed intracellular peptide drug with gold nanoparticle-peptide hybrids. *Nanoscale.* **7** (29), 12356-12360, doi:10.1039/c5nr02377g, (2015).

24 Kimling, J. *et al.* Turkevich method for gold nanoparticle synthesis revisited. *The Journal of Physical Chemistry B.* **110** (32), 15700-15707, doi:10.1021/jp061667w, (2006).


3. Line 79: This was not tested in this article. Please avoid making claims that are unsupported.

4. Line 87: I’m not sure why you say this when you did not demonstrate any in vivo applications in here.

Response: The objective of JoVE is to present the protocols. We have now added clear references to declare previous work. We clearly stated that this

article is for the formulation protocol. I hope this will be acceptable by the reviewer.

5. Line 97: I edited for clarity, please verify If this is correct.
Response: Thanks. It is correct.

6. Line 108: Mention sonicator frequency and amplitude (available in the manufacturer datasheet)
Response: Its frequency is 40MHz. Our device does not have a parameter to change intensity. Its datasheet does not mention its amplitude. I added this information into the text.

7. Line 122: In the introduction you call this GNP-PKCi, please be consistent in the terminology!

Response: I now call our product as GNP/PKCi.

8. Line 143: How do you decide this? Please explain.

Response: I explained it on a note below。

Note: The GNP pellet contains 6.3x $10^{11}$ particles. For example, we administrated 1.3x $10^{12}$ particles in 500 $\mu$L of 0.9% NaCl to treat rat IR injury. In that situation, we added 232 $\mu$L of 0.9% NaCl to each of three pellets. After pooled them together, we collected 500 $\mu$L of GNP/PKCi solution.

9. Line 154：This is incorrect, OD measurement does not reveal any about the biophysics of the GNP/PKCi hybrid.

Response: I have changed it to “solubility of”

10. Line157: Can you add additional assessment steps? Eg. Those you have mentioned here: http://www.rsc.org/suppdata/c5/nr/c5nr02377g/c5nr02377g1.pdf. They don’t need to be detailed, you can mention them and cite references.

11. Line 159: 1 test on 3 samples is really insufficient for the results section of a scientific publication. Please add additional figures and results discussion to discuss the assessments, in vivo tests, drug delivery, cellular uptake, microscopy you performed etc.

Response: We have changed the title of the paper as “formulation” only. Therefore, we do not need to refer too much on the previous publication.

12.Line164: Can you show a curve?

13. Line170: What is the reference that you are comparing these curves against, i.e. how do you know this is good quality?

14. Line171: What is an example of bad preparation?

Response: I now answer to all these questions. I added new figures to show how we know good or bad quality.