

Video Article

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

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## Abstract

Protein kinase C-delta inhibitor (PKC $\delta$ i) 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 PKC $\delta$ i. 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 PKC $\delta$ i (GNP/PKC $\delta$ i) are stable in distilled water, 0.9% NaCl, and phosphate-buffered saline containing bovine serum albumin or fetal bovine serum. Intravenous injection of GNP-PKC $\delta$ i was previously shown to prevent ischemia-reperfusion injury of the lung. This article outlines a protocol to formulate GNP/PKC $\delta$ i and assess the physicochemical properties of GNP/PKC $\delta$ i. 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<sup>1</sup>. However, serious complications after lung transplantation remain an obstacle. In the early stages following lung transplantation, primary graft dysfunction is the most harmful complication<sup>1</sup>, and its primary cause is ischemia-reperfusion (IR)-induced acute lung injury<sup>2</sup>.

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<sup>3</sup>. 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 C $\delta$  inhibitor (PKC $\delta$ i) has been used in the heart, brain and lung<sup>4,5,6,7,8</sup>. These studies showed that PKC $\delta$ i decreased inflammation and apoptosis during reperfusion. It has also prevented pulmonary IR injury in rats and in a lung transplant model<sup>9</sup>. PKC $\delta$ i 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<sup>9,10,11</sup>. Nanoparticles, small particles ranging from 1 to 100 nm in diameter<sup>12</sup>, have been explored as candidates in facilitating drug delivery<sup>13</sup>. In particular, gold nanoparticles (GNPs) are regarded as noninvasive and nontoxic. Therefore, we have developed GNPs as drug delivery carriers for peptide-based drugs<sup>14,15</sup>.

The surface of GNPs can be manipulated for specific applications such as molecular recognition<sup>16,17</sup>, chemical sensing<sup>18</sup>, imaging<sup>19</sup>, 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<sup>14</sup>. The cysteine (C) residue at the N terminus of these peptides contains a thiol group that can conjugate to the gold surfaces<sup>14</sup>. This peptide/GNP hybrid was further used to deliver PKC $\delta$ i (CSFNSYELGSL). The optimized molar ratio of P2:P4 to PKC $\delta$ i is 47.5:2.5:50. GNPs conjugated with PKC $\delta$ i (GNP/PKC $\delta$ i) are stable in distilled water, 0.9% NaCl, and phosphate-buffered saline (PBS) containing bovine albumin or fetal bovine serum<sup>14</sup>. Intravenous injection of GNP/PKC $\delta$ i has been shown to prevent ischemia-reperfusion injury of the lung<sup>15</sup>. This article outlines a method to formulate GNP/PKC $\delta$ i and describes how to evaluate the physicochemical properties of GNP/PKC $\delta$ i. We have used similar methods to formulate other peptide-based drugs conjugated to GNP<sup>20,21,22</sup>. We hope this article will draw more attention to this novel formulation for intracellular drug delivery.

## Protocol

### 1. Preparation of Peptide Solutions

1. Retrieve the peptides (P2: CAAAAE, P4: CAAAAW, PKC $\delta$ i: 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.

2. Weigh 0.01 g of each peptide on a microscale. Put each peptide into a separate 50 mL conical tube.
  3. Add 18.74 mL of deionized (DI) water to the P2 tube.
  4. Add 16.93 mL of DI water to the P4 tube.
  5. Add 8.21 mL of 50% acetonitrile diluted in DI water to the PKC $\delta$ i tube.
  6. Vortex the peptide solutions briefly. Put the 50 mL conical tubes in a sonicator (40 MHz) for 5 min.
  7. Bring the peptide solutions to a biosafety cabinet. All peptide solutions prepared should be 1 mM.
  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 PKC $\delta$ i tube, such that each solution is diluted to 50  $\mu$ M and stored in its own tube.
  9. Aliquot 1 mL of each 50  $\mu$ M peptide solution into 1.5 mL microtubes.
  10. Place all aliquots in a -80 °C freezer.
- NOTE: Stock solution should be remade each month.

## 2. Formulation of GNP/PKC*i*

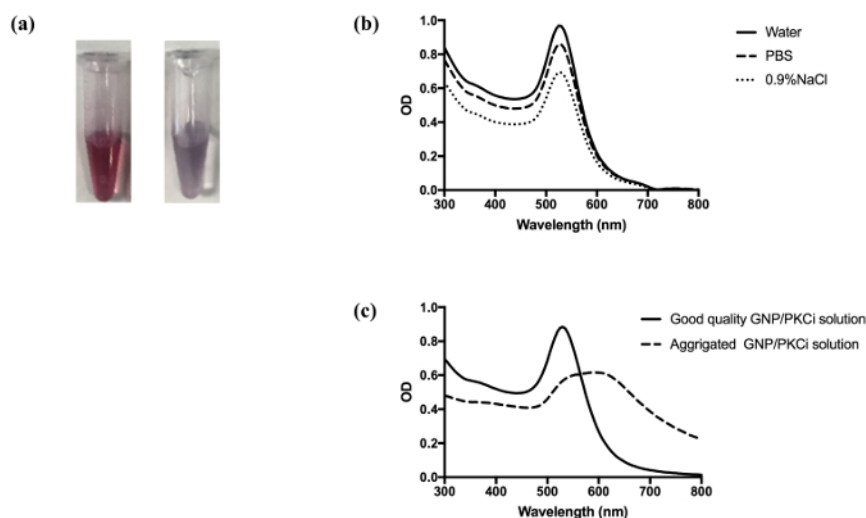
1. Remove the peptide solutions from the -80 °C freezer. Thaw them at RT. Bring them to a biosafety cabinet.
  2. Add 475  $\mu$ L of P2, 25  $\mu$ L of P4, and 500  $\mu$ L of PKC $\delta$ i solution to 9 mL of 20 nm GNP solution ( $7.0 \times 10^{11}$  particle/mL) in a 15 mL tube.
  3. Exit the biosafety cabinet. Wrap the 15 mL tube with aluminum foil. Leave it on a shaker at RT overnight.
  4. Return the samples to the biosafety cabinet. Aliquot 1 mL of GNP/PKC*i* into each 1.5 mL microtubes.
  5. Centrifuge the tubes in a micro-centrifuge for 30 min at  $15,294 \times g$  at 4 °C.
  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.
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/PKC*i*, 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  $\mu$ L of 0.9% NaCl, we add 232  $\mu$ L of 0.9% NaCl to each of three pellets. After pooling them together, we can then collect 500  $\mu$ L of GNP/PKC*i* solution.
- NOTE: Mix the desired solvent well before diluting the GNP/PKC*i* pellet, otherwise the GNP/PKC*i* will aggregate.

## 3. Assessment of GNP/PKC*i* Hybrid Solubility

1. Pour 0.5 mL of GNP/PKC*i* solution into an acryl cuvette. Place the acryl cuvette on a UV-Vis spectrophotometer and test the peak absorption<sup>15</sup>.

### Representative Results

Care should be taken to evaluate the biophysical properties of the GNP/PKC*i* 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/PKC*i* 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,  $\Delta$ optical density ( $\Delta$ OD = OD at 525 nm - OD at 440 nm) decreases (**Figure 1C**).



**Figure 1: Quality of GNP/PKC*i*.** (A) Properly prepared GNP/PKC*i* is pink in color (left). Aggregated GNP/PKC*i* appears light purple (right). (B) Good GNP/PKC*i* 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/PKC*i* preparations. When the GNP was aggregated, the peak of absorption was shifted to the right. Moreover,  $\Delta$ OD decreased. [Please click here to view a larger version of this figure.](#)

## Discussion

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

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<sup>24</sup>. Usually, smaller GNPs can deliver drugs into cells more efficiently than larger ones, since they can more easily diffuse into their target regions<sup>25</sup>. Second, GNPs are non-toxic *in vitro* and *in vivo*<sup>26</sup>, rendering them safe drug carriers. Third, hydrophobic drugs can be loaded onto the modified GNPs<sup>27</sup>. 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<sup>14</sup>, stabilize them in physiological conditions, and impart new bioactivities<sup>15</sup>. The peptides were thoughtfully designed, with three regions including gold binding, spacing, and functional regions<sup>14</sup>. 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<sup>14</sup>. The ratio between PKC $\delta$ i and P2/P4 peptides was also systemically tested and selected<sup>15</sup>.

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<sup>28,29,30</sup>. So far, this formula has only been tested in cell cultures and small animal models<sup>15</sup>. 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.

## Disclosures

The authors have nothing to disclose on this project.

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