NCL Method PCC-16

Quantitation of PEG on PEGylated Gold Nanoparticles Using Reversed Phase High Performance Liquid Chromatography and Charged Aerosol Detection

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This protocol assumes an intermediate level of scientific competency with regard to techniques, instrumentation, and safety procedures. Rudimentary assay details have been omitted for the sake of brevity.

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1. Introduction

This protocol describes a method for the quantitation of polyethylene glycol (PEG) in PEGylated colloidal gold nanoparticles using reversed phase high performance liquid chromatography (RP-HPLC) with charged aerosol detection (CAD). The method can be used to calculate the total PEG on the nanoparticle, as well as the bound and free unbound PEG fractions after a simple centrifugation step. This is a significant distinction as the bound PEG fraction affects biocompatibility, circulation time, and overall nanoparticle efficacy. PEG quantitation can be achieved through two methods, one involving dissolution of colloidal gold nanoparticles by potassium cyanide (KCN) and the other by displacement of PEG by dithiothreitol (DTT). The methods outlined herein were applied to 30 nm colloidal gold grafted with 20 kDa PEG, but they can be easily adapted to any size colloidal gold nanoparticle and PEG chain length.

The method development was previously published in ref. (1) and the detailed protocol is published in ref. (2).


2. Principles

Understanding the nanoparticle surface is one of the challenges in nanoparticle characterization, yet it is an important feature to measure because it defines the nanoparticles' biocompatibility (3-6). For example, colloidal gold nanoparticles are often surface functionalized with the biocompatible, hydrophilic polymer poly(ethylene) (PEG, i.e. PEGylation) to reduce opsonization, increase circulation half-life, and provide stability by preventing aggregation as a result of its neutral charge (7-11). Physicochemical characterization techniques such as UV-Vis spectroscopy for gold nanoparticle concentration, dynamic light scattering for hydrodynamic size (NCL Protocol PCC-1), and zeta potential analysis (NCL Protocol PCC-2) indicative of surface charge are commonly employed to characterize PEGylated colloidal gold nanoparticles. These techniques can qualitatively assess the presence of PEG but are not sensitive enough to distinguish differences in PEG quantity, density, or presentation.

To address this characterization gap, two methods have been developed which allow for the quantitative measurement of PEG on PEGylated gold nanoparticles (**Figure 1**) (1). In the first method, referred to as the displacement method, dithiothreitol (DTT) is used to displace PEG

from the gold surface. Centrifugation pellets the DTT-coated gold nanoparticles while the supernatant contains the excess DTT and dissociated PEG, which are further separated using RP-HPLC. In the second method, referred to as the dissolution method, potassium cyanide (KCN) is used to dissolve the gold nanoparticles and liberate the PEG. Excess CN^-, $Au(CN)_2^-$, and free PEG are separated using RP-HPLC. In both methods, detection of the PEG is accomplished via CAD after RP-HPLC separation. A centrifugation step prior to either of the two methods can be used to separate free PEG from bound PEG (**Figure 2**). The displacement and dissolution methods are outlined here using 20 kDa PEGylated 30 nm colloidal gold nanoparticles but can be extended to other size colloidal gold nanoparticles and PEG chain lengths (1).

A. Displacement Method

B. Dissolution Method
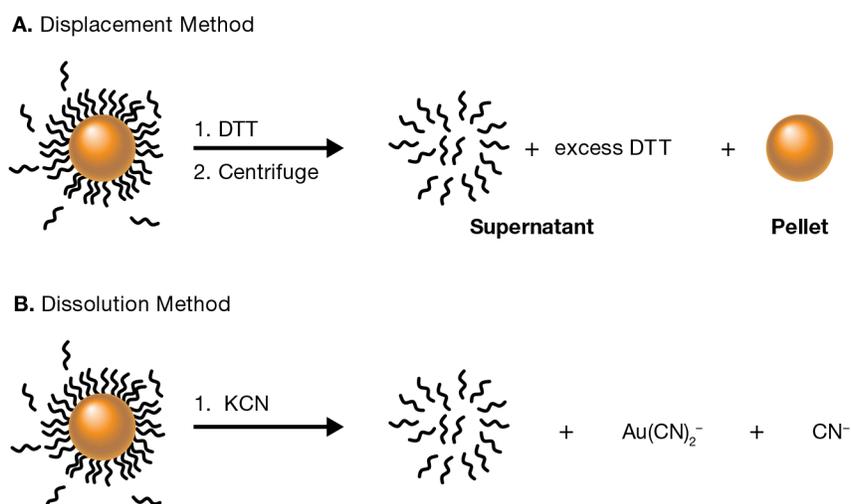
Figure 1. Displacement and dissolution techniques to quantitate the total (bound and free) PEG on AuNPs. RP-HPLC with CAD is used for both techniques to quantitate the PEG coating. A) The displacement method requires excess DTT to displace PEG from the gold nanoparticle surface. After centrifugation, the displaced PEG and excess DTT make up the supernatant while the gold nanoparticles form a pellet. B) The dissolution method dissolves gold nanoparticles with the addition of potassium cyanide (KCN). RP-HPLC separates the PEG component. Reproduced with permission from reference (1).
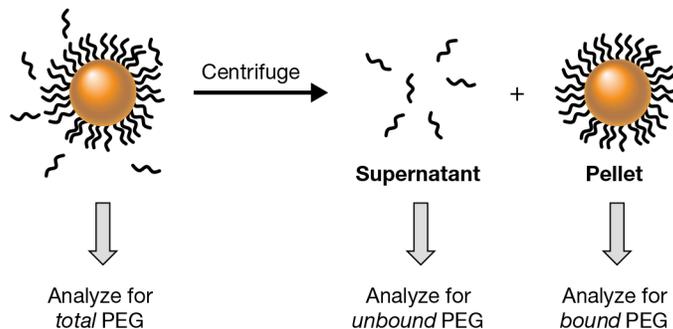
Figure 2. Separation method to quantitate bound and unbound PEG on AuNPs. A centrifugation step of the PEGylated AuNPs forms a fraction of the unbound PEG in the supernatant and the AuNP-bound PEG in the pellet. Then, PEG can be quantitated for each of these populations by RP-HPLC with CAD. Reproduced with permission from reference (1).

3. Reagents, Materials, and Equipment

Note: The NCL does not endorse any of the suppliers listed below; their inclusion is for informational purposes only. Equivalent supplies from alternate vendors can be substituted.

    3.1   Reagents

        3.1.1   Acetonitrile with 0.14% (w/v) trifluoroacetic acid, HPLC grade.

        3.1.2   Water with 0.14% (w/v) trifluoroacetic acid, HPLC grade.

        3.1.3   1 M potassium cyanide (KCN) in water, HPLC grade.

                Caution: Always wear appropriate personal protective equipment and take precautions throughout this procedure. Be especially careful when handling KCN as it is extremely toxic. Follow your lab safety protocols for handling and disposing of such chemicals.

        3.1.4   550 mM dithiothreitol (DTT) in water, HPLC grade, make fresh as required.

    3.2   Materials

        3.2.1   PEGylated (20 kDa) colloidal gold nanoparticles (AuNP); 50 µg/mL gold concentration.

3.2.2 Free 20 kDa PEG, ideally from the same lot of PEG used in the nanoparticles.

3.3 Equipment

3.3.1 RP-HPLC system consisting of a degasser, capillary pump, well-plate autosampler, PLRP-S column (100 Å, 4.6 mm ID×150 mm, 5 μm), and charged aerosol detector (CAD).

4. Experimental Procedure

4.1 Dissolution Method

4.1.1 Sample Preparation for Total PEG

4.1.1.1 Add 10 μL of 1 M KCN solution to 100 μL PEGylated AuNP. Here, the solution represents a 10-fold molar excess of KCN relative to 50 μg/mL AuNPs, the stock concentration used in our samples. The red solution (AuNP) will turn clear after several minutes of vortexing, signaling the end of the dissolution process. Be sure the sample has completely turned clear prior to injection. Typically, this color change occurs within 20 minutes of incubation with KCN.

Caution: Always wear appropriate personal protective equipment and take precautions throughout this procedure. Be especially careful when handling KCN as it is extremely toxic. Follow your lab safety protocols for handling and disposing of such chemicals.

4.1.2. Sample Preparation for Unbound and Bound PEG

4.1.2.1 Centrifuge 200 μL of the PEGylated AuNP sample for 30 minutes at 14,000 rpm and 26°C, yielding a red pellet.

Note: If the amount of bound PEG falls below the LLOQ and there is no detectable free PEG, the sample will need to be concentrated appropriately to increase signal strength. In order to do this, centrifuge the sample down at 14,000 rpm, 25°C for 30 minutes and then remove a known volume of supernatant. For example,

spin down 300 µL of sample and remove 150 µL of supernatant. This will concentrate the sample 2-fold and thus increase signal strength. This can be done as many times as necessary to bring the sample to a concentration that falls roughly in the middle of the calibration standards range. As long as the 1:10 ratio of KCN or DTT to PEGylated AuNP is maintained, any appropriate volumes may be used to meet sample injection requirements. Be sure to correct for concentration during data analysis.

4.1.2.2 Remove the supernatant and reserve for HPLC analysis to test for free unbound PEG.

4.1.2.3 Record the pellet volume for each sample (typically 6-15 µL) and add the appropriate volume of water to give a total volume ranging from 50-100 µL. Resuspension volumes vary to meet the detection limits of the RP-HPLC CAD system. The pellet fraction is analyzed for bound PEG concentration by HPLC.

4.1.2.4 Add 10 µL of 1 M KCN solution to the re-suspended pellet. Vortex sample until it turns clear. Test for bound PEG.

4.2 Displacement Method

4.2.1 Sample Preparation for Total PEG

4.2.1.1 Add 10 µL of 550 mM DTT solution to 100 µL of PEGylated AuNP. Here, the sample represents a near 1000-fold excess of DTT relative to PEG. Vortex the sample thoroughly. A minimum of five minutes for incubation is ample time to allow the PEG to be displaced from the surface of the AuNP.

4.2.1.2 Vortex sample and centrifuge for 30 minutes at 14,000 rpm and 26°C, yielding a red pellet (AuNPs). The clear supernatant, containing displaced (bound) PEG and any free unbound PEG, is retained for HPLC analysis.

4.2.2 Sample Preparation for Unbound and Bound PEG

4.2.2.1 Centrifuge 200 µL of the PEGylated AuNP sample for 30 minutes at 14,000 rpm and 26°C, yielding a red pellet. Please see the Note in Section 4.1.2.1.

4.2.2.2 Remove the supernatant to test for free unbound PEG.

4.2.2.3 The pellet fraction is analyzed for bound PEG concentration. Record the pellet volume for each sample (typically 6-15 µL) and add water for a final volume range of 50-100 µL. Resuspension volumes vary to meet the detection limits of the RP-HPLC CAD system.

4.2.2.4 Add 10 µL of 550 mM DTT solution to the re-suspended pellet. Vortex the sample thoroughly. Incubate sample for a minimum of five minute to allow the PEG to be displaced off the surface of the AuNP.

4.2.2.5 After addition of DTT, vortex the sample and centrifuge for 30 minutes at 14,000 rpm and 26°C, yielding a red pellet (AuNPs). The clear supernatant, containing displaced (bound) PEG, is reserved for HPLC analysis.

4.3 Prepare PEG Calibration Standards

4.3.1 Prepare a set of PEG calibration standards in HPLC grade water based on the lower limit of quantification (LLOQ) and limit of detection (LOD). When determining the LLOQ, construct a calibration curve and probe the lower end until a concentration is reached that does not fall in line with the rest of the curve. The point on the curve above this one is the LLOQ. To determine the LOD, inject consecutively lower concentrations of PEG until there is no apparent peak. The lowest concentration that produces a visible peak is the LOD. Calibration standards are typically prepared at concentrations ranging from 2.5 – 60 µg/mL in HPLC grade water. In order to fall in the linear range on the calibration curve, standards usually will fall somewhere between 2.5 and 60 µg/mL. This range will also vary instrument to instrument so be sure to test the LLOQ and LOD to construct a proper calibration curve. In addition, signal strength will vary

slightly with PEG molecular weight due to peak broadening for the lower weight PEG chains. For example, 2 kDa and 5 kDa PEG will require a slightly higher calibration range than 10 kDa and 20 kDa PEG. Also note that both methods of PEG quantitation would most likely work for any molecular weight PEG, but was only tested here for 2, 5, 10, and 20 kDa. A minimum of seven standards are recommended.

  4.3.2 Mix 100 µL calibration standard with 10 µL 1 M KCN. Standard samples are prepared fresh and used immediately.

 4.4 RP-HPLC Conditions

  4.4.1 The essential component of the chromatographic system needed for PEG quantitation is a charged aerosol detector (CAD). The CAD is operated with a fixed drift-tube temperature of 35°C. The nebulizer gas consists of compressed nitrogen with a flow rate of 1.68 L/min and pressure of 35.1 psi.

  4.4.2 The mobile phase consists of water/acetonitrile (A/B, HPLC grade, 0.14% (v/v) trifluoroacetic acid).

  4.4.3 The elution gradient for 10 kDa PEG is 30% B for 3 min, ramp to 50% B in 20 min, hold at 50% B for 3 min, and ramp down to 30% B in 3 min. The elution gradient for the 5 kDa PEG was 30% B for 3 min, ramp to 50% B in 10 min, hold at 50% B for 3 min, and ramp down to 30% B in 3 min. The elution gradient for the 2 kDa PEG was 30% B for 3 min, ramp to 50% B in 5 min, hold at 50% B for 3 min, and ramp down to 30% B in 3 min.

  4.4.4 The injection volume is 40 µL and the flow rate is 1 mL/min.

5. Data Analysis

 5.1. Open the elution profiles of the PEGylated AuNP (**Figure 3**) as well as those of the standards.
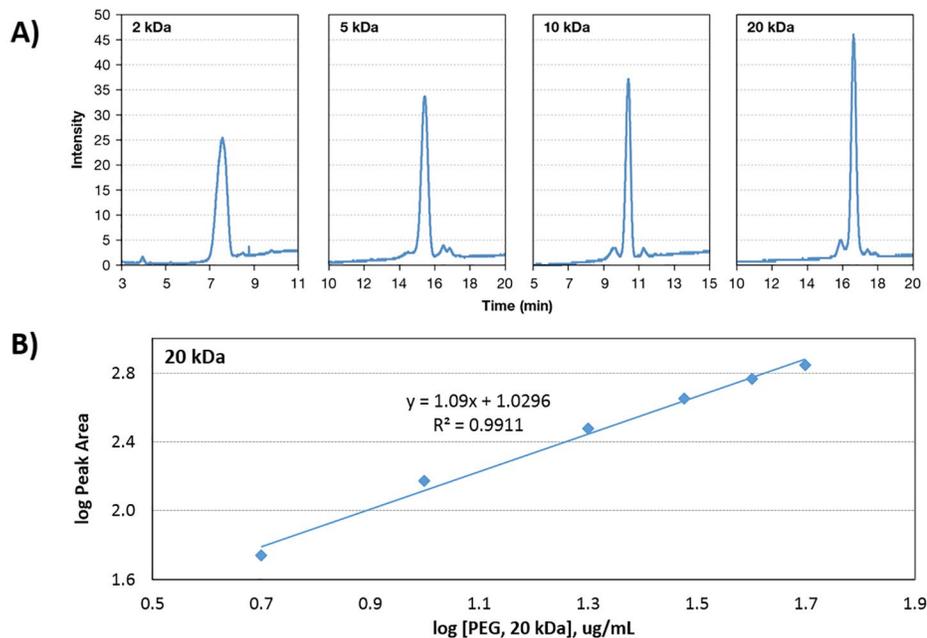
Figure 3. A) RP-HPLC chromatograms with CAD of 2-, 5-, 10- and 20 kDa mPEG-SH. B) 20 kDa mPEG-SH standard calibration curve. The PEG samples include 50 mmol/L DTT. Separation and quantitation was performed on an RP-HPLC system (Agilent G4225A, Palo Alto, CA) with a capillary pump (Agilent G1312B), well-plate autosampler (Agilent G1329B), Agilent PLRP-S column and CAD (ESA Corona Ultra). Analysis was performed on a Corona Ultra CAD instrument and Agilent Chemstation. Figure A) Reproduced with permission from reference **(1)**.

5.2.　Integrate the PEG peak area for each sample.

5.3.　Create a calibration curve plotting each calibration standard's peak area versus concentration. If the curve is nonlinear, plot log peak area against log of the PEG concentration and use this graph to quantitate the amount of PEG in each sample. While this log-log analysis is more traditional in regards to CAD response, one may be able to plot peak area against PEG concentration on a linear scale with a lower range of standards (in our case 2.5-25 µg/mL) for sample PEG quantitation.

6. References

1. Smith MC, Crist RM, Clogston JD, & McNeil SE (2015) Quantitative analysis of PEG-functionalized colloidal gold nanoparticles using charged aerosol detection. *Anal Bioanal Chem* 407(13):3705-3716.

2. Smith MC & Clogston JD (2017) PEG Quantitation Using Reversed Phase High Performance Liquid Chromatography and Charged Aerosol Detection. *Characterization of Nanoparticles Intended for Drug Delivery,* Methods in Molecular Biology, ed McNeil SE (Humana Press), 2nd Ed Vol 1682.

3. Albanese A, Tang PS, & Chan WCW (2012) The Effect of Nanoparticle Size, Shape, and Surface Chemistry on Biological Systems. *Annu Rev Biomed Eng* 14:1-16.

4. Harris JM & Chess RB (2003) Effect of pegylation on pharmaceuticals. *Nat Rev Drug Discov* 2(3):214-221.

5. Jokerst JV, Lobovkina T, Zare RN, & Gambhir SS (2011) Nanoparticle PEGylation for imaging and therapy. *Nanomedicine-Uk* 6(4):715-728.

6. Yowell SL & Blackwell S (2002) Novel effects with polyethylene glycol modified pharmaceuticals. *Cancer Treat Rev* 28:3-6.

7. Almeida JP, Figueroa ER, & Drezek RA (2014) Gold nanoparticle mediated cancer immunotherapy. *Nanomedicine-Uk* 10(3):503-514.

8. Blanco E*, et al.* (2011) Nanomedicine in cancer therapy: innovative trends and prospects. *Cancer science* 102(7):1247-1252.

9. Cai W, Gao T, Hong H, & Sun J (2008) Applications of gold nanoparticles in cancer nanotechnology. *Nanotechnology, science and applications* 2008(1).

10. Jain S, Hirst DG, & O'Sullivan JM (2012) Gold nanoparticles as novel agents for cancer therapy. *The British journal of radiology* 85(1010):101-113.

11. van Vlerken LE & Amiji MM (2006) Multi-functional polymeric nanoparticles for tumour-targeted drug delivery. *Expert opinion on drug delivery* 3(2):205-216.

7. Abbreviations

AuNP	Gold nanoparticle
CAD	Charged aerosol detector
DTT	Dithiothreitol
KCN	Potassium cyanide
LLOQ	Lower limit of quantification
LOD	Limit of detection
PEG	Polyethylene glycol
RP-HPLC	Reversed phase high performance liquid chromatography