Report

Formation of Protein Coronas and Consequent Effect on Nanotoxicity of Cobalt Nanoparticles

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Metal-on-metal hip arthroplasty (MoMHA), consists of mainly cobalt(Co)/chromium(Cr) alloy of the prostheses, has been developed and been applied rapidly worldwide. However, concerns have arisen as the devices are causing severe tissue and bone damage in some patients. Pseudotumor, a soft-tissue mass formation with pain and discomfort has been identified, which is in correlated with Co/Cr nanoparticle release from the wear of the prostheses. The mechanism of pseudotumor formation is still unknown. The aim of the project is to analyse the composition of protein coronas on commercially available cobalt nanoparticles, and to observe that whether the formation of protein corona plays a role in macrophage recognition, phagocytosis and cytotoxicity. Commercially available Co nanoparticles will be used for this experiment. Particle size, number, surface area and zeta potential will be measured. The particles will then be incubated with: (1) normal human serum (2 ml each from three healthy individuals); and (2) normal human synovial fluid (1 ml each from three healthy individuals), at a concentration of 1×10^{12} NP/ml (table 1). After the corona formation, the proteins on part of the particles will be extracted for analysis. Also effects of protein corona formation on macrophage recognition and phagocytosis in addition to cytotoxicity will be studied.

Assessment of cytotoxicity, in particular the effect of nanoparticles on cells are an important measure to evaluate the potential application of nanomaterials in medicine. One type of clinically relevant nanotoxicity is the metal nanoparticles, mainly cobalt produced in wear of hip prostheses. These particles can cause catastrophic soft tissue responses, such as tissue necrosis, inflammation and fibrosis in some patients. However, the mechanism is still poorly understood.

In order to study the mechanism, large number of dose dependent assessments on a variety of cells and different pathway analysis of each type of cells are required. There are available biochemical and morphological assays; however, it is very difficult to quantify morphological results using current approaches, such as confocal microscopy.

High throughput imaging system provides a powerful tool to perform both morphological and quantitative analysis. We have had a GE InCell2000 system which has the potential of high throughput analysis, but a suitable standard protocol and analysing platform are needed for the cytotoxicity study. These protocols have been well established in Dublin. It is expected to be invaluable to learn and set up similar system in Swansea through collaboration.

We expect to reveal the protein corona formation and protein corona composition of cobalt nanoparticles in human serum and synovial fluid. The hypothesis is that protein corona can form on cobalt nanoparticles, and the formation of protein corona should change the behaviour of metal nanoparticles, in particular the consequent macrophage recognition, phagocytosis and cytotoxicity.

Materials and Methods

1. Corona study

(1) Stock Co nanoparticles were 102 mg/ml Co NP in ethanol. These NPs were sonicated at power 60 for 5 minutes, and diluted 1:10 into deionised water.

(2) Human plasma, pooled from 6 donors, and human synovial fluid pooled from 3 donors, were centrifuged at 16,000 g × 3 min to pellet any particles.

(3) For corona formation, Co NPs were incubated with 10% plasma (mimic the NPs in tissue), 55% plasma (mimic the NPs in blood) and 90% synovial fluid (mimic the NPs in joint cavity), at 37°C for 1 hour and shake at 250rpm to reduce sediment.

(4) After incubation, the NPs were centrifuged at 16,000g, 15°C for 20 min, and washed 3x in PBS, followed by centrifugation at 16,000g, 15°C for 10 min.

(5) At the end of wash, SDS loading buffer was added, and denature at 100°C × 5 min.

(6) Electrophoresis using 4% gel.

(7) Silver staining.

2. Cytotoxicity assay

(1) THP-1 cells were chosen for cytotoxicity assay.

(2) Cells were stimulated for macrophage differentiation with 100nM PMA for 3 days, before seeding in 96 well plates at IMM, St James Hospital. The seeding density of THP-1 cells were 2×10^4 cells/well

(3) Cells were culture for 2 days before the Co NPs were added.

(4) Cells were divided into 4 groups, control, Co NPs without corona (three subgroups, 10^{10}, 10^{11}, 10^{12} NPs/ml), Co NPs with corona formation in 10% human plasma (three subgroups, 10^{10}, 10^{11}, 10^{12} NPs/ml), and Co NPs with corona formation in 55% human plasma(three subgroups, 10^{10}, 10^{11}, 10^{12} NPs/ml).

(5) After the Co NPs were added, cells were cultured for 24 and 48 hours before fluorescent staining and imaging by InCell1000.

(6) For fluorescent staining, cells were washed in PBS before stained using three fluorescent dyes, Calcein-AM 0.5 μl/ml, EthD-1 (2 μl/ml) and Hoechst 33324 (1 μl/ml) in PBS. The concentration were optimised using InCell 2000. Cells were stained for 15 minutes at 37° for 15 minutes, washed in PBS before imaging.

Results

1. Protein corona formation

The electrophoresis of proteins extracted from the Co-NPs after incubated in human plasma and synovial fluid is shown in Figure 1. It demonstrated that there were protein corona formation on CoNPs incubated with 10% and 55% human plasma, and 90% human synovial fluid.

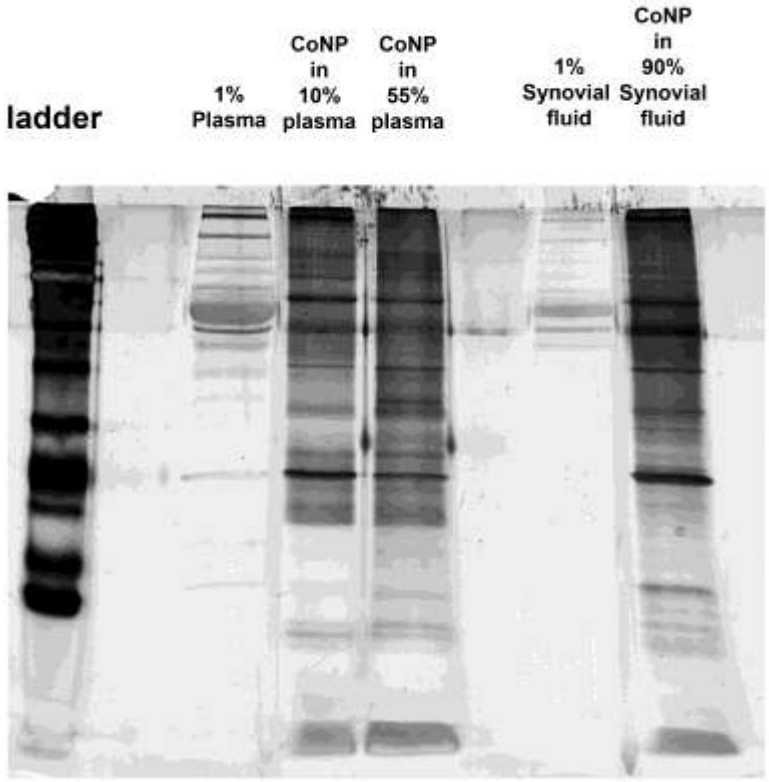

Figure 1. Protein corona formation on CoNPs in 10% and 55% human plasma, and 90% human synovial fluid.

2. Cytotoxicity of CoNPs with/without corona formation

In order to explore the cytotoxic effect of cobalt nanoparticles *in vivo* a macrophage like cell line was exposed to cobalt nanoparticles for 24 and 48hrs. THP-1 cells were incubated with varying concentrations of naked particles, particles incubated in 10% serum and particles incubated in 55% serum to replicate exposure of the particles to tissue and blood respectively. After 24hrs exposure the cells were stained with Hoechst 33342 (1ul/ml PBS), Calcein (0.1ul/ml PBS) and ETHD-1 (1ul/ml PBS) to label all cells, live cells and dead cells respectively.

Cytotoxicity was assessed using the High Content Analysis (HCA) method employed at Trinity College Dublin. The INCELL 1000 (Dublin) acquired images in 3 channels for subsequent analysis (Swansea). Images were acquired in the blue, green and red emmision spectra indicative of total cell number, live and dead cells respectively. The Developer software suite was used to segment objects in each channel and count the number of events in each field. The number of dead cells was expressed as a percentage of the total cell number. Total cell number was calculated by addition of live and dead cell events. 7 non-overlapping fields within each well ensured 90% of the well was imaged and no events were replicated. There were 5 replicate wells per treatment grouping.

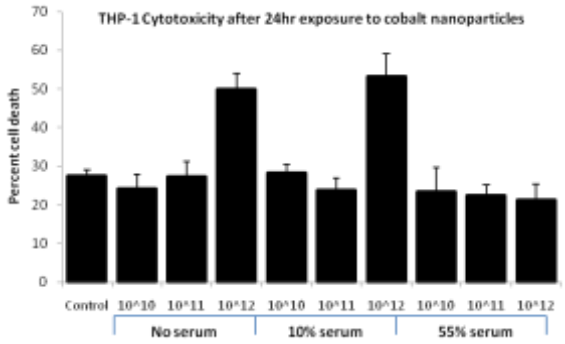

Figure 1. The effect of 24hr exposure to cobalt nanoparticles

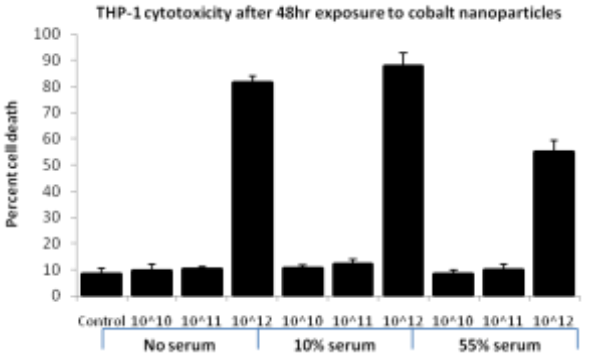

Figure 2. The effect of 48hr exposure to cobalt nanoparticles

After 24hrs exposure to nanoparticles (figure 1) the cytotoxicity remained at control levels in the 10^{10} and 10^{11} concentration particle applications regardless of any prior serum incubation. However cytotoxicity increased at the 10^{12} treatment in the naked particles and the 10% serum particles but this effect appeared to be negated by prior particle incubation in 55% serum. In this group cytotoxicity was maintained at control levels.

After 48hr exposure to nanoparticles (figure 2) the cytotoxicity increased under all treatments relative to control. The 10^{12} nanoparticle treatment did not image to a standard that allowed subsequent analysis in the live cell channel. It was not possible to undertake a count that represented the true number of live cells and a small number of live cells resulted in a disproportional percentage of dead cells, hence these data are omitted. However at the lower concentrations of nanoparticle exposure we see a dose dependent response whereby cytotoxicity increases in line with increasing concentration. No serum effect or decrease in cytotoxcity has been observed at the 48hr timepoint as was present after 24hr exposure.

Conclusion and future work

As predicted protein corona formation were proved when CoNPs were incubated with human plasma and synovial fluid. The cytotoxicity assay showed that CoNPs at 10^{12}/ml were toxic to THP-1 cells, which is consistent with our previous finding. Interestingly, when corona formed on CoNPs in 10% plasma, the cytotoxicity was similar in comparison with CoNPs without corona formation; however, when corona formed on CoNPs in 55% plasma, the cytotoxicity was significantly reduced at both 24 and 48 hours.

There are certain limitation of these work. Firstly, the communication between the research group at IMM, Trinity and us was not organised properly, which caused difficulty of the preparation of cytotoxicity assay. Since the THP-1 cells were carried from Swansea, although the cells were survived, the viability of control cells were not good enough for the experiment. Secondly, the staining optimised in InCell 2000 was not suitable for InCell1000. The Hoechst 33342 staining was very faint, and unable to show strong signal for counting total cell numbers. For Calcein staining, there was some background noise from the plate and nanoparticles. Also, as human synovial fluid arrived one day after the cytotoxicity assay, it was not possible to test the cytotoxicity of this group. Finally, there should be other markers which could be used to understand more of the mechanism in addition to cytotoxicity.

For immediately future work, we need well controlled experimental condition to confirm the differences of the corona formation between 10% and 55% plasma, and 90% synovial fluid. It will be better to compare them at the same concentration of protein level. As mass spectra was not used in

the experiment, we have no convincing data to show what the protein corona compositions are. Therefore we need MS analysis to map the protein compositions.

From the cytotoxicity assay, it is very interesting to demonstrate that protein corona formed in high concentration of plasma actually reduce the cytotoxicity of CoNPs. We still do not know if this is also true for synovial fluid, as any nanoparticles produced in hip replacement will firstly in contact with synovial fluid. As these particles will eventually phagocytosed by macrophages, according to our previous study, it will be important to understand the biological significance of the corona formation on macrophage recognition.

Appendix: Protocols to identify protein corona formation on cobalt nanoparticles

Materials

1. Cobalt nanoparticles
2. Human plasma, pooled of plasma from 6 donors (supplier?)
3. Synovial fluid (Cambridge Bioscience), pooled from 3 donors.
4. Sonication (FisherBrand, FB11002)
5. Heating device (Grand-Bio, PHMT, SC20)
6. Centrifuges (Eppendorf, Centrifuge 5810R)
7. Gel preparation
8. Acrylamide/Bis-Acrylamide (40% solution, Sigma, A7802)
9. Electrophoresis (BioRad,
10. Silver staining (Cosmo Bio Co.Ltd, 2D-Silver Staining II)
11. Scanning of gel staining

Detailed protocol

Protein corona formation

1. Thaw human plasma to RT and centrifuge it at 16,000 g for 10 minutes, to ensure removal of any particles in serum.
2. Stock Co NP is 100 mg/ml in ethanol. Dilute stock Co NP 1:10 in deionised water, and sonicate for 5 minutes (power 70×5min).
3. Prepare Co NP in 10% (similar to tissue exposure) and 55% (similar to blood exposure) human plasma.
4. CoNPs in 100% synovial fluid.
5. Incubation for 1 hour at 37°C, mix them a few times (shake at 250rpm does not suspend the particles) during incubation.
6. Centrifuge at 16,000 g × 20 minutes. Carefully remove supernatant. Make sure no visible particles can be seen in supernatant. To confirm if there are still NPs in liquid, the supernatant may be centrifuged at full speed for 90 minutes to check if there are remaining particles.
7. Resuspend Co NPs in PBS, care should be taken to make sure proper resuspension of the NPs (sediments are visible if not suspended enough; however, NPs may bind to tips and stain the tips dark if it is suspended for too long. Sonication will detach protein corona from the NPs. Any suggestions?).
8. Centrifugation at 16,000 g × 10 minutes
9. Repeat 7-8, ×3
10. Add SDS loading buffer
    (1) SDS loading buffer (3×, Biolabs, #7703S)
    (2) Dilute SDS loading buffer, 20 µl to 40 µl PBS
    (3) Add 1µl of DTT into 10 µl of diluted SDS loading buffer
    (4) 60 µl loading buffer per sample
11. Boil for 3 min at 100°C

Protein corona detection

1. Gel preparation

Choose 1 mm gel plate to make gel, as 1.5 ml gel will be too thick to run the samples.

	10% Gel		**4% Gel**
(1) MilliQ H_2O	3.095 ml		2.82 ml
(2) 1.0M Tris-HCl pH 8.9	3.75 ml	0.4M Tris HCl, pH 6.9	1.57 ml
(3) Acrylamid/Bis	2.5 ml		500µl
(4) 10% Ammonium persulfate	50 µl		50µl
(5) Temed	5 µl		5 µl

Prepare 10% gel for better separation. For gel preparation a few things are important: 1) make sure the sponge at the base of the gel plate is dried properly, if not the gel may leak out; also make sure the two glass plates are placed in the correct place, leave no gap or misalignment which gives gap and causes leaking; 2) when load the glass plates to the gel caster, handle the glass plates with care in case breakig the glasses; 3) fill the solution to a certain level, leave room for 4% gel (at the lower level of the upper green plastic frame); 4)use 1 ml Gilson pippetman tip to fill the gel plates; 5) add 2-propanol on the top to press the gel, which will keep the top of gel to be a straight line and smooth surface; dispose of 2-propanol before loading the 4% gel; 6) the gel will set in 20 minutes; 7) after the 10% gel is set, dispose the 2-propanol and fill in 4% gel, load comb for sample loading, continue to fill the gel and drive away any bubbles formed; 8) wait until the 4% gel is set.

2. SDS-PAGE Electrophoresis

SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis, is a technique widely used to separate proteins according to their electrophoretic mobility (a function of length of polypeptide chain or molecular weight). SDS gel electrophoresis of samples that have identical charge per unit mass due to binding of SDS results in fractionation by size.

Prepare 1× electrophoresis running buffer from 10× stock solution (what it is?). Voltage 130 mV

3. Silver staining

Follow the manufacturer's manual from (Cosmo Bio Co.Ltd, 2D-Silver Staining II)