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Time evolution and dynamic cellular uptake of PEGYlated gold nanorods

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The protein corona formed and evolved when PEGYlated gold nanorods (GNRs) are cultured with cells. The changes of protein corona alter nanorods characteristics significantly which results in dynamic cellular uptake pathways. This dynamic nature should be well considerded for future applications and the prevention of data misinterpretation.

Of the vast amounts of nanoparticles, GNRs have received particular attention due to their potentials in cancer therapy, drug delivery, hyperthermia and bio-imaging.[1] The most common method to produce GNRs is wet chemical seed-mediated synthesis in the presence of cetyltrimethylammonium bromide (CTAB).[2] After the centrifugation process, there is still much CTAB adsorbed on the GNRs surface which is toxic to cells. So far, there have been many molecules to replace CTAB, of which thiolated PEGs belong to the most widely used molecules.[3] PEGYlated GNRs exhibit high colloidal stability and biocompatibility which are more suitable for further clinical application.[4]

When exposed to cell culture medium, the nanoparticles will adsorb many kinds of proteins forming protein corona, which change their characteristics and become their new identities.[5] It is known that PEGYlated GNRs can largely reduce the adsorption of serum proteins, yet the formation of protein corona could not be avoided.[6] Though the initial protein corona of PEGYlated GNRs contains few proteins and has little effect on the characteristics of PEGYlated GNRs, the status of PEGYlated GNRs after culturing with cells for dozens of hours is largely unknown. The data is indispensable for a better understanding of the biological fate of PEGYlated GNRs.

To exert their potential in drug delivery, cancer therapy and other functions, GNRs need to be internalized by cells. Understanding the cellular uptake mechanisms through which PEGYlated GNRs are transported to cells is of great importance to improve delivery strategies and therapeutic efficiency. Previous investigations reveal the possible internalization pathways when GNRs are exposed to cells initially, while no study has ever investigated whether the cellular uptake pathways will change in different time. The formation of protein corona is a dynamic process and the biological identities of nanoparticles changes consequently.[7] As the characteristics of GNRs alter time dependently, it is reasonable to hypothesize that the internalization of GNRs may also be a dynamic process.[8-10] A better understanding of the cellular uptake pathways will help to better comprehend nano-bio interactions and prevent data misinterpretation.

We synthesized GNRs using seed-mediated growth method with minor revision and modified them with mPEG5000-SH (Fig. S1).[8] Cell cytotoxicity of PEGYlated GNRs on six cell lines was evaluated initially. The data indicated that PEGYlated GNRs could selectively suppress vascular related cells (Fig. S2A-C) proliferation while had no effect on normal cells (Fig. S2D-F), which suggested that PEGYlaed GNRs could be a potential agent for angiogenesis therapy. We choose human retinal endothelial cells (HREC) for subsequent experiments and the concentration of 50 μg/mL of PEGYlated GNRs was used.

Both cell culture system and biological system in vivo are sustained with complex nutrition fluids which maintain a dynamic status through continual interaction with cells or tissues. A recent investigation indicates that the newly synthesized and secreted proteins by the cells could change the protein corona composition of nanoparticles,[11] while it is unknown whether PEGYlated GNRs are able to keep their initial state for a long time, especially in the surrounding of dynamic fluid. To study the protein corona of PEGYlated GNRs in different times, we observed the protein corona using transmission electron microscope (TEM). The protein corona could be seen clearly under TEM images (Fig. 2A, B, C). We then performed SDS-PAGE to separate the proteins covered

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PEGYlated GNRs after culturing with HREC for 1h, 24h and 48h. The data showed that more proteins were adsorbed on

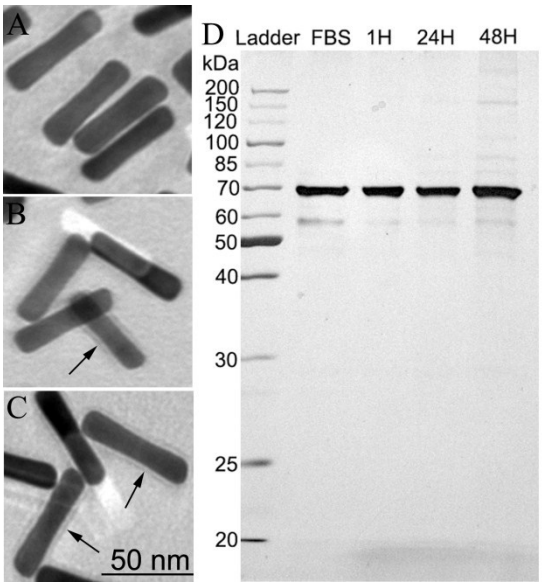

Fig.1 *The evolution of protein corona on PEGYlated GNRs. TEM images of PEGYlated GNRs cultured with cells for (A) 1 h, (B) 24 H and (C) 48 h. The black arrows indicated the protein corona. (D) Silver stained SDS-PAGE of serum proteins adsorbed on PEGYlated GNRs.*

PEGYlated GNRs when the nanorods were cultured with cells for a longer time (Fig. 2D). The large number of proteins covered PEGYlated GNRs might have significant effects on the characteristics of nanorods. To better understand the composition of the protein corona, we performed liquid chromatography-tandem mass spectrometry (LC-MS) to identify these proteins. The data showed that as time went on, more protein counts were found and the species of most abundant proteins had changed (Table. S1, S2, S3). The bovine serum albumin was one of the most abundant proteins at different times, while more human proteins could be detected as time went by. There were proteins secreted by the cells as basement membrane-specific heparan sulfate proteoglycan core protein, thrombospondin-1 and annexin a2. There were also cytoskeleton proteins such as Myosin-9, actin and Filamin A, which might be carried by the exocytotic nanorods when they were excreted by cells.

After PEGYlated GNRs were exposed to the cell culture medium, the protein corona were formed immediately and kept changing, which was not only because of their dynamic exchange of proteins with cell culture medium but also due to the dynamic exchange of molecules between the medium and the cells. The adsorption and dissociation occurs constantly, and equilibrium could be reached in the biological fluid without living cells or tissues. However, when it comes to the living system, the situation may be quite different. On one hand, the cells consume and secret molecules constantly. On the other hand, the endocytosis and exocytosis of nanorods by cells occur at the same time.[12] Both the secreted molecules and exocytotic nanorods could affect the composition of protein corona, which may alter the identity of PEGYlated GNRs significantly.

To further study the changes of PEGYlated GNRs culturing with cells for different times, we observed the shape of nanorods with TEM. There were apparent aggregations when the nanorods were exposing for a long time (Fig. 2A, B, C). The result of UV-visible spectrophotometry was consistent with TME as the surface plasmon resonance band was wider with obvious red shift time dependently (Fig. 2D). Aggregation usually occurs when the van der Waals attractive forces between nanorods are greater than the electrostatic repulsive forces produced by the surface of nanorods[13]. The high

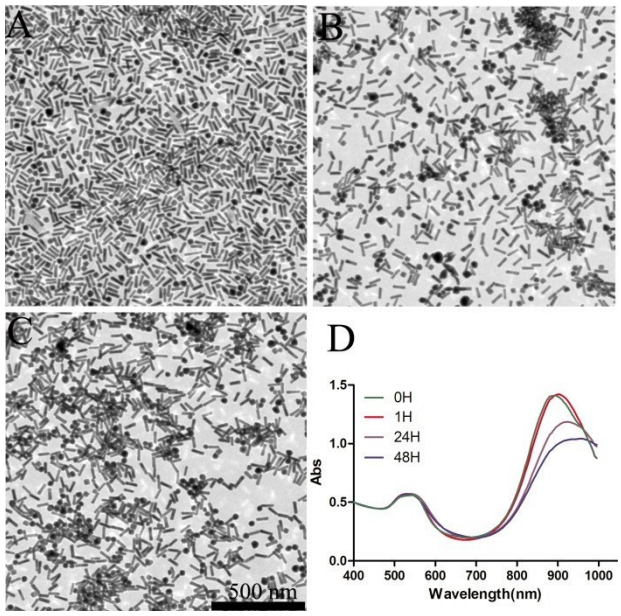

Fig. 2 *The changes of the characteristics of PEGYlated GNRs. TEM images of PEGYlated GNRs cultured with cells for (A) 1 h, (B) 24 H and (C) 48 h. (D) UV-Vis-NIR absorption spectra of indicated PEGYlated GNRs.*

intense of protein corona could cause a thermodynamically favoured replacement of surface-modified molecules with serum proteins, which were able to de-stabilize nanoparticles[14]. Meanwhile, it was also possible that the composition of protein corona was affected by the aggregation of nanorods, as the mechanism of endocytosis and exocytosis of aggregated nanorods might be different from the previous ones. These data suggested that the characteristics of PEGYlated GNRs changed time dependently, and this dynamic nature must be considered fully to understand their interaction with cells better.

Cellular uptake of GNRs is considered to be one of the vital processes to fully exert their functions. The well-recognized endocytic pathways that internalize nanoparticles are macropinocytosis, clathrin-mediated endocytosis and lipid raft-mediated endocytosis.[15] Macropinocytosis is a process that cells trap large fluid (>1μm) pockets by formation and enclosure of membrane protrusions[15, 16]. Clathrin-mediated endocytosis occurs following the signal by transmembrane receptors, which then forms “coated pits” by the assembly of cytosolic proteins and clathrin is the major assembly unit. The diameters of clathrin-coated pits are in the range of ~150 nm and this process usually internalizes particles of that size[15, 16].

Lipid-raft mediate endocytosis is characterized by lipid rafts which are plasma membrane microdomains enriched in cholesterol and sphingolipids involving in the compartmentalization of molecules, ligands, receptors and viruses. These lipids are small (10~200nm) and could internalize particles within these sizes[15, 16]. Previous investigations mainly focus on the initial cellular internalization pathways after GNRs being exposed to the cells for hours.[8, 10] However, whether the cellular uptake pathways change and how they change when the characteristics of GNRs evolved with time are unknown.

We investigated the cellular incorporation of PEGYlated GNRs within the cells at different time. The data indicated that PEGYlated GNRs could be internalized by HREC and mainly located in endosomes. The nanorods cultured with cells for 24h or 48h usually presented in aggregated manner in cells (Fig. S3). To better investigate the endocytic pathway of PEGYlated GNRs in HREC cells, we used both chemical inhibitors and siRNAs to block cellular uptake pathways and then assessed internalized PEGYlated GNRs by cells using inductively coupled plasma mass spectrometry (ICP-MS).

Inhibitor concentrations were determined according to the toxicity assay (Fig. S4). The concentrations for Chlorpromazine (CPZ), Methyl-β-cyclodextrin (MβCD) and 5-ethyl-N-isoproppylamiloride (EIPA) were 50μM, 5mM and 100μM separately. These inhibitors are classical pharmacological inhibitors that targeted three major endocytic pathways as clathrin-mediated endocytosis (CPZ), macropinocytosis (EIPA) and lipid raft-mediated endocytosis (MβCD) separately. The data showed that PEGYlated GNRs were internalized by HREC mainly through lipid-raft mediated endocytosis initially (Fig. 3A). After culturing with cells for24h, PEGYlated GNRs were internalized still mainly through lipid-raft mediated endocytosis, while clathrin-mediated endocytosis and macropinocytosis also participated in (Fig. 3B). After 48h culturing with cells, macropinocytosis became the mainly cellular uptake pathway though lipid-raft mediated endocytosis and clathrin-mediated endocytosis also played a role (Fig. 3C).

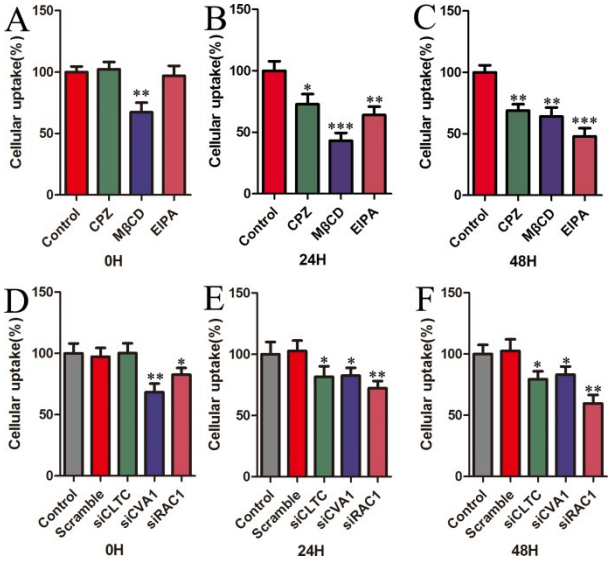

Fig.3 *Dynamic cellular uptake of PEGYlated GNRs. Cellular uptake pathways of indicated PEGYlated GNRs into HREC cells were determined by endocytosis inhibitors and siRNAs. Data represent the means ± SD of three separate samples (*, p<0.05, **, p<0.01, ***, p<0.001).*

Furthermore, we assessed the cellular uptake pathways by siRNA knockdown targeting clathrin heavy chain (CLTC), caveolin-1 (CAV1) and Ras-related C3 botulinum toxin substrate 1 (RAC1). The knockdown efficiency of the siRNAs were determined by Western blot assay(Fig. S5). These three molecules are well known to play key roles in clathrin-mediated endocytosis, caveolin-mediated endocytosis (belonging to lipid raft-mediated endocytosis) and macropinocytosis. The data were basically consistent with the results of those using chemical inhibitors. Initially, caveolin-mediated endocytosis was the major one through which PEGYlated GNRs was internalized (Fig.3D). As time went by, macropinocytosis became the major one (Fig.3E,F). While, different from the data of chemical inhibitors, macropinocytosis played a role all the time and the changes between three groups at different time using chemical inhibitors were more apparent than using siRNAs. The reason might be that siRNAs usually targeted at specific internalized pathways but chemical inhibitors usually had additional effects except targeting specific pathways.

The changes in endocytic pathways might largely result from the changes of PEGYlated GNRs. On one hand, the composition of protein corona altered in different times, which could partially account for the changes. What the cells “saw” were not the nanorods but the nanorods-protein corona complexes once the nanorods were coated with protein corona[5]. As the composition and quantity of protein corona changed overtime, the nanorods-protein corona complexes might be recognised by different receptors on the cells, which could mediate cellular uptake of nanorods in different pathways. On the other hand, the increasing aggregation of PEGYlated GNRs could be another reason as large subjects usually were internalized through macropinocytosis. These results strongly suggested that the dynamic nature of cellular uptake must be taken fully into account in the design of study to prevent data misinterpretation, especially in the investigation of drug delivery and photothermal therapy.

In conclusion, we synthesized GNRs using seed-mediated growth method and modified them with mPEG5000-SH successfully. The cytotoxicity of PEGYlated GNRs on HREC was assessed and the concentration of 50μg/mL was chosen for consequent experiments. PEGYlated GNRs were co-cultured with HREC for 1h, 24h and 48h separately. The proteins were adsorbed to the surface of PEGYlated GNRs forming protein corona, which altered significantly in different time leading to increasing aggregation of the nanorods. These apparent changes of the PEGYlated GNRs could affect the internalization pathways suggesting a dynamic nature of the cellular uptake pathways in cell culture medium with living cells. The conclusion drew in our study is not only restricted to PEGYlated GNRs as most nanoparticles exposing to living cells or tissues would face the dynamic nature of biological fluids

which will lead to the dynamic identities of nanoparticles. Understanding the dynamic nature of the bio identities and cellular uptake pathways of the nanoparticles is of great significance for the comprehension of nano-bio interactions and for the design of study to prevent data misinterpretation.

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Table of content

The protein corona formed and evolved of PEGYlated GNRs which alter GNRs characteristics significantly leading to dynamic cellular uptake pathways.