

Sensitivity of detection by biological AFM for gold-labeled liposomes at human coronary artery endothelial cell (HCAEC) membranes

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ABSTRACT

Biological processes such as endocytosis/exocytosis, interactions between cells and the dynamic reorganization of the cytoskeleton are between the topics extensively studied using the Atomic Force Microscopy (AFM) technique. The topographical structure of the cell membrane itself is complex, rich in natural corrugations and ridges. These surface characteristics obscure the identification of extracellular structures when using AFM for membrane imaging. We have endeavored the utilization of 90nm nano-gold particles to label functionalized liposomes and monitor their endocytosis process on HCAEC. We successfully established a suitable and systematic method to study the internalization mechanism, binding sites, and distribution of gold labeled liposomes as a novel delivery system on endothelial cells.

Our findings also demonstrated the usefulness of the AFM technique, combined with fluorescent microscopy, to investigate liposome detection within the membrane surface of endothelial cells and elucidate their binding mechanism.

Keywords: liposomes, AFM, endocytosis, gold particles, HCAEC

1 INTRODUCTION

Engineering of non-toxic nanoparticles for tissue-specific targeting has gained widespread attention this decade. Of particular interest is the development of biologically active nanoparticles that can facilitate the delivery of therapeutic and/or diagnostic agents to precise regions within the human body. While many have successfully designed and tested such “nanovectors” in vivo, little is known about how these particles dynamically interact with cell membranes. Atomic Force Microscopy (AFM) is a surface analytical technique that generates nano-scale topographic images under physiological conditions [1].

Consequently, AFM combined with fluorescence microscopy, is an attractive tool for interrogating nanoparticle:cell membrane interactions and may afford the opportunity to image biological processes, such as cellular endocytosis of nanovectors and reorganization of the cytoskeleton, in real-time.

Liposomes were one of the earliest classes of engineered nanoparticles to be utilized for the purpose of delivering drugs within the human body. The ability to pegylate liposomes for the purpose of mitigating a systemic immune response, targeting specific tissues, and/or enhance prolonged vascular circulation has made these particles attractive nanovectors to increase the efficacy of anti-cancer drugs. One of the major disadvantages of using standard liposome formulations is their rapid clearance from circulation due to uptake by the reticulo-endothelial system (RES). To resolve this problem, long circulation liposomes have been developed since the early 1990's [3], [6]. Long-circulating liposomes have polyethylene glycol (PEG) derivatives attached to their surfaces. The conformational flexibility of PEG chains creates a sterical barrier that allows the liposomes to evade the RES uptake and remain in circulation for a longer time increasing the possibility for targeting [2].

Our laboratory is interested in designing liposomes that are immunospecific to inflamed endothelium, a hallmark of atherosclerosis, for the purpose of imaging lipid-rich plaques prior to the onset of clinical symptoms (eg, angina, coronary stenosis, etc.). While in vivo studies of plaque-specificity (imaged via computed tomography, CT) are currently underway, we are also focused on understanding how these liposomes interact with endothelial cells that have been induced into an inflammatory response.

Some nanoparticles can also be used as imaging contrast agents and as reinforcement elements to improve visual enhancement [7]. The properties of colloidal gold have provided excellent detection capabilities for single-molecule tracking [5]. The main aim of this project is to standardize the use of gold particles as a non-toxic means

to detect immuno-liposomes on the membrane of living cells to elucidate internalization processes and binding efficiency on Human Coronary Artery Endothelial Cells (HCAEC) using AFM.

2 RESULTS AND DISCUSSION

Initial attempts to monitor the endocytosis process of functionalized liposomes on HCAEC resumed in the need of an imaging contrast agent to improve visual enhancement during AFM imaging. FITC labeled liposomes were not detectable during AFM scanning when incubated on HCAEC. Positive fluorescence signaling was detected due to the fluorescently labeled lipid but these liposomes were basically lost on the surface of the cell membrane when scanning using the AFM technique. This difficulty arose with the concept of using the properties of colloidal gold to perform single-molecule tracking, which has been very popular for morphological studies.

2.1 AFM analysis of liposomes

AFM is a high resolution technique that demonstrated the structural properties of non-gold labeled and gold labeled liposomes. To guarantee the preservation of the original structure, both types of liposomes were analyzed after fixation with 10% formalin and deposited on fresh cleaved mica. The AFM was operated in Tapping mode on air and RTESP tips during liposome scanning. The AFM section analysis for non-gold labeled liposomes reported an average size of 121.5nm. This result was comparable to the measurements initially collected with the DLS (129nm).

Once the 90nm gold particles were covalently linked to the liposomes, samples collected from the stock solution demonstrated (via AFM imaging) the formation of gold-liposome complexes dispersed on clusters about 303nm in size. We observed the geometrical structure of typical gold-liposome clusters composed of several particles with individual diameters of 98, 80 and 73nm, as seen in the AFM sectional analysis. According to DLS measurements, these gold-liposome complexes had an average size of 285nm.

2.2 Endocytosis and liposome coupling

The surface topology and characteristics of biological membranes can routinely be described using biological AFM instruments, which allow scanning in physiological conditions. Nevertheless, the application of this technique has rarely been approached to resolve kinetics of liposome cell uptake. To investigate how the endocytosis process takes place within the cell, we used 90nm gold particles to track FITC labeled liposomes [4].

Sequential images (**Figure 1**) were taken to 4 different incubation times (15, 30, 60 and 120 min) to describe how the gold-liposomes were basically penetrating the plasma membrane. We used fluorescence imaging to routinely identify the positive signalling emitted by the FITC labeled liposomes and locate the cells to be selected for scanning.

The **Figure 1 (A-B)** shows a HCAEC incubated for 15min with FITC labeled liposomes and conjugated with nano-gold particles. At this stage of the process, the liposomes were physically attached to the cell membrane. They quiver when probed by the AFM tip during the scanning, but the gold-liposomes were already strongly bonded to the cell. It was observed that the liposome clusters started to internalize after the 30min incubations (images not shown). The same **Figure 1 (C-D)** also illustrates the endocytosis process occurring at the 60min incubation. In this image is clearly appreciated how the plasma membrane is taking over the extracellular material, which will gradually engulf into the cell. The corresponding fluorescence image **Figure 1 (D)** shows a strong positive signalling of the two gold-liposomes clusters (indicated with arrows) selected for scanning. Observations were also taken in HCAEC incubated for 120min finding that the gold-liposomes almost internalized totally after this period of time as observed in the **Figure 1 (E)**. These series of images demonstrated that the gold-liposome uptake took place in a time interval of 120min, summarising the endocytosis process in the following terms: liposome attachment to the plasma membrane (15-30min), internalization (30-60min) and membrane fusion (120min). These results strongly agree with the findings reported by Ramachandran *et al.*, (2006) [8], whom studied the endocytosis process with cisplatin-encapsulated liposomes.

On the other hand, the analysis of negative controls (HCAEC incubated with gold-liposomes that gave negative signaling in the fluorescence imaging), observed smooth and even membrane surfaces as appreciated. Signs of elevated or raised areas were not present during AFM scanning and the lack of fluorescence signaling indicated the absence of FITC labeled gold-liposomes.

3 CONCLUSION

The use of 90nm colloidal gold particles permitted the localization of FITC coupled liposomes to study internalization kinetics using AFM. We were able to demonstrate the mobility of the liposomes through the cell membrane prior absorption. It was determined that the endocytosis process occurs in the following terms: liposome attachment to the plasma membrane (15-30min), internalization (30-60min) and membrane fusion

(120min). We found that gold-liposome clusters could efficiently be taken through endocytosis by the cell membrane regardless of their geometrical structure.

The gold-liposomes used in this study physiologically behaved as expected when exposed to an endocytosis inhibitor (Dynosore) to block their internalization process. Therefore, the gold nanoparticles did not influence in any means the liposome uptake. The versatility of the AFM was also demonstrated to detect nano-biomolecules in complex systems. We established in this research a potential method to track biological materials. The 90nm colloidal gold nanoparticles are a non invasive contrast agent that efficiently improves the visual enhancement when using AFM to study biological nanoprocesses.

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Figure 1.- AFM images (A, C and E) and corresponding bright field images (B, D and F) of HCAEC incubated for 15 (A-B), 60 (C-D) and 120min (E-F) with FITC labeled liposomes conjugated with 90nm gold particles. The cells showing positive signaling in the bright field images were selected and scanned using a Bioscope II. The AFM image obtained for a HCAEC incubated for 15min (A) shows a gold liposome cluster attached to the cell that shivered to probe contact. (C) The AFM image of a HCAEC incubated for 60min with gold liposomes and scanned to 45 μ m² clearly shows the internalization process occurring at this stage. The AFM micrograph of a HCAEC incubated for 120min (E) corroborated that the gold-liposomes have almost completely internalized at this point giving good positive signaling in the bright field image (F). Cells fixed in formalin 10% and scanned in Contact mode in liquid (DNP-S probes, $f_0=12-24$ kHz, $k=0.06$ N/m).

