

# A “leukolike” multistage delivery system to overcome biological barriers

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## ABSTRACT

The localization of theranostic nanoparticles (NPs) to a tumor site has been the subject of considerable research that so far has not translated into comparably comforting advances in clinical medicine.

Due to limitations in their structure and surface properties, NPs are still unable to overcome the multiplicity of biological barriers they encounter after intravenous administration and that adversely impact their ability to reach the intended target at effective concentrations.

We developed a novel hybrid biomimetic delivery system that combines together the characteristics of an artificial multistage delivery system with the physiologic components that trigger the natural ability of leukocytes (T and B cells) to transmigrate from the blood stream to the tumor site.

**Keywords:** nanoparticles, delivery system, leukocytes, biological barriers

## 1 INTRODUCTION

The understanding of the mechanisms that affect the bio-distribution of an injected agent has been the objective of extraordinary amounts of research. Results reported in the literature amply demonstrate that only a small fraction of infused drugs and imaging agents reach the desired target upon successfully overcoming all the encountered biological barriers.

The blood-brain barrier, the intestinal lumen endothelium, or the vessel endothelial walls are prime examples of physical biological barriers to injected agents. The effectiveness of therapeutic and contrast agents are also hampered by the reticulo-endothelial system (RES) that is comprised of macrophages and scavenger endothelial cells that reduce the circulation time and availability of most of the currently developed drug delivery systems [1].

In the last decade, the drug delivery field has been revolutionized by the ability to create NPs that can encapsulate several kinds of therapeutic and imaging agents. Nanoporous Silicon Particles (NSPs) have been proposed as ideal delivery systems for the targeted release of theranostic agents to tumor sites as porous silicon is biocompatible, biodegradable and easy to functionalize [2-4]. The ability to tune particle size and shape as well as

porosity and pore size results in increased efficiency of drug loading while the coating of the NSP surface with stimuli-responsive polymers allows for the controlled release of the payload thus enabling environmentally triggered delivery [5,6]. Even though polymer-grafted particles exhibit prolonged residency times in the blood several studies have indicated association of opsonins with stealth particles and a fraction of intravenously injected long-circulating NPs are rapidly deposited in the liver and the spleen [7-9].

In this work we present a new class of biomimetic multidrug delivery systems able to avoid clearance by the reticulo-endothelial system (RES), to increase the transcytosis through the endothelial barrier and to reduce the immune system response while targeting the tumor site.

In order to achieve these aims we took advantages from the natural ability of leukocytes to transmigrate from the blood stream into the surrounding inflammatory tissues and we assembled a system composed of NSPs preloaded with diagnostic and therapeutic nanoparticles (NPs) [10], and coated with the cellular membranes of primary leukocytes isolated from the peripheral blood of mice. This leukocyte like system, named “leukolike”, is the first attempt to transfer the leukocyte transendothelial migration ability on a manmade particle.

## 2 EXPERIMENTAL METHODS

### 2.1 NSPs Fabrication

NSPs with the desired size, shape and porosity were prepared following the protocols previously developed in our laboratory [11] (Figure 1a).

### 2.2 Cell Culture and Plasma Membranes Isolation

Primary leukocytes were grown in RPMI-1640 medium containing 10% fetal bovine serum (FBS), supplemented with 1% glutamine.  $2.8 \times 10^8$  cells were enucleated in a hand-held Dounce homogenizer using an appropriate homogenization buffer (HB). The homogenized cells were laid on the top of a discontinuous sucrose density gradient (55/40/30%, w/v) and the plasma cellular membranes isolated by ultracentrifugation. After ultracentrifugation ten

fractions were collected from the top to the bottom of the gradient and their protein content analyzed by dot blot.

### 2.3 Protein Characterization

Monoclonal antibody (mAb) against CD45 and Lck (lymphocyte cell specific protein tyrosine kinase) were used as specific markers for the cellular membrane, while nucleoporin p62 and COX-IV were used as markers for the nuclear and mitochondrial membranes respectively. Lymphocyte function-associated antigen-1 (LFA-1) and CD3z content was also investigated since they are involved in the leukocytes' biological functions (Figure 2). The fractions containing both the cellular markers and the functional proteins were pooled together, washed and stored in a saline solution and later used for the NSPs surface coating.

### 2.4 Membrane Coated NSPs Preparation and Characterization

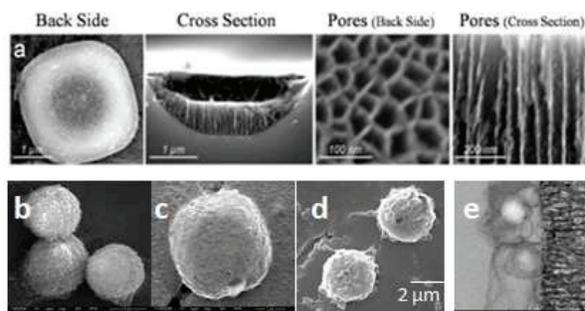
NPSs ( $\sim 1.5 \times 10^6$ ) with a diameter of  $2.8 \mu\text{m}$  were modified with aminopropyltriethoxysilane (APTES) and incubated with the isolated cellular membranes over night at  $4^\circ\text{C}$ . The hybrid system was characterized by transmission electron microscopy (TEM) and scanning electron microscopy (SEM). The protein composition of the hybrid system surface was also analyzed by fluorescence activated cell sorting (FACS). FITC- and APC-conjugated monoclonal antibodies against CD3z and LFA-1 (also known as CD11a) were used for FACS analysis.

## 3 RESULTS AND DISCUSSION

We first attempted to isolate the plasma cellular membranes of primary leukocytes isolated from the peripheral blood of mice, through a discontinuous density sucrose gradient. Primary leukocytes are homogenized in a complete HB by a hand hold dounce homogenizer. The cellular lysate is separated by the cellular debris by centrifugation at a low speed, laid on a discontinuous sucrose density gradient and ultracentrifuged. After ultracentrifugation the distribution of specific protein markers associated to the different kinds of cellular membranes (nuclear, mitochondrial, plasma membranes) was investigated by dot blot over ten fractions collected from the top to the bottom of the gradient. The plasma cellular membrane enriched fractions were therefore identified (Figure 2).

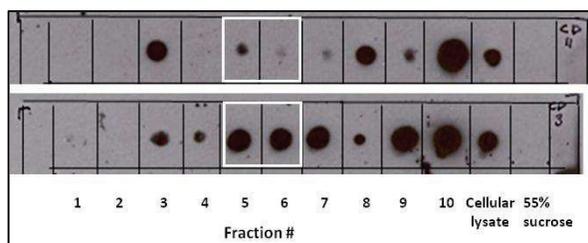
In an aqueous solution, the isolated leukocyte cellular membranes spontaneously organize into multilayer vesicles with a variable diameter due to the hydrophobic interactions among the lipid tails. During the incubation with the NSPs the lipid vesicles were disrupted and fused around the NSPs as a consequence of their interaction with the surface of the NSPs [12]. By applying TEM and SEM (Figure 1 b-e) we observed that leukocyte membranes

consistently and stably adhered around the NSPs, showing a resemblance between the leukocytes and our hybrid delivery system (hence the name "leukolike").



**Figure 1:** SEM images of NSPs (a) leukocytes (b) and NSPs coated with leukocyte membranes (c, d). Detailed TEM image of leukocyte cellular membranes coating a NSP (e).

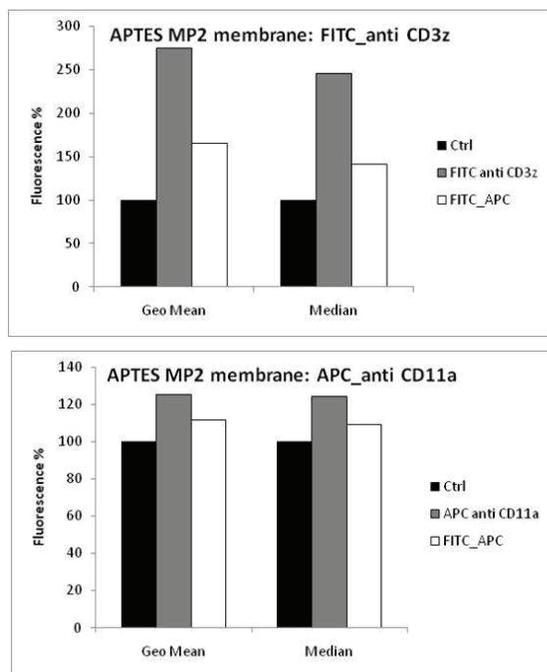
Since our "leukolike" system is the first challenge to build biological functions on NSPs, we particularly focused on the characterization of the amount of both LFA-1 and CD3z proteins retained in the assembled system. FACS analysis showed that these proteins were still exposed on the surface of the "leukolike" system (Figure 3). CD3z is a protein associated with the T cell receptor that is responsible for leading the leukocytes tropism towards the tumor site, while LFA-1 is an integrin physiologically expressed on leukocytes membranes which mediates the transendothelial migration from the blood stream into the surrounding tumor tissue [13].



**Figure 2:** Dot blots relative to LFA-1 and CD3z identification (top and bottom respectively). The dots in the white boxes represent the fractions containing the leukocytes cellular membrane used for the NSPs coating.

In fact it is well known that during an inflammatory event leukocytes are recruited to the inflammatory lesion by crossing the endothelial wall through paracellular and transcellular routes [14]. Independently of the route taken, leukocytes transendothelial migration (TEM) is triggered by the interaction between the intercellular adhesion molecule-1 (ICAM-1) on the endothelial membrane and LFA-1 on the leukocyte membrane. Interaction with ICAM-1 triggers the activation of endothelial intracellular signaling pathways that result in extensive cytoskeletal remodeling

events that alter endothelial cell contractility and function, facilitating leukocyte diapedesis. During TEM, endothelial cuplike structures enriched in ICAM-1 and LFA-1 surround the site of diapedesis and allow the leukocytes to squeeze through the tight junctions, as they migrate towards the interstitial tumor space [15, 16].



**Figure 3:** FACS analysis of CD3z and LFA-1 (CD11a) expression on NSPs coated with leukocyte membranes. Results show that, after the coating, Cd3z and LFA-1 are still present on the surface of the “leukolike” system.

Significantly, TEM does not require any molecular activation in the leukocytes aside from the remodeling of the cytoskeleton to fit the channel that is formed in the endothelial cell. Therefore, TEM is predicted to occur upon contact with leukocyte membrane and does not require an active participation of the leukocytes. Moreover the overall dimensions of the NSPs are already below the size of the transmigratory channel and can effectively cross the endothelial cell boundaries.

#### 4 CONCLUSIONS AND FUTURE PERSPECTIVE

These results demonstrated the feasibility of building a delivery platform that combines the characteristics of an artificial delivery system (agents loading and release, biodegradability and biocompatibility) with the natural properties of leukocyte cells (free circulation in the blood stream, transendothelial migration and tropism towards the tumor site). The purpose of this hybrid system is to enhance

the probability of delivering a theranostic payload to the intended target at effective concentrations.

In order to guarantee the targeting ability of the “leukolike” system, the leukocytes will be *ex vivo* expanded and genetically modified to express a chimeric antigen receptor (CAR) with specificity for a desired tumor antigen. The plasma membranes from the autologous, minimally manipulated, *ex vivo* expanded leukocytes, will be isolated and used for the coating of the NSPs. By exploiting the potential of leukocytes to migrate to the tumor microenvironment, we expect that our “leukolike” system will make contact with the endothelial cell luminal surface, will undergo transmigration by displaying the LFA-1 protein and will accumulate in the tumor microenvironment through the CAR tropism.

The ability of the “leukolike” system to escape RES sequestration and to undergo endothelial transmigration will be verified *in vitro* with primary macrophages and with a model of endothelial barrier.

By combining the understanding the features and characteristics of natural processes with emerging nanotechnologies, we envision to create a hybrid delivery system capable of mimicking the natural course of actions of a complex biological “machine” such as the leukocyte. This system would combine for the first time the advantages of both technology and biology.

In conclusion, the advantages of this biomimetic system are: 1- increased circulation time and prevention of RES uptake by shielding the delivery vector with cellular membranes of autologous leukocytes; 2- decrease of the immune reaction against the components of the delivery system; 3- prevention of the release of the preloaded theranostic NPs in the vasculature; 4- increase of the accumulation of the delivery system at the target site.

Finally, since leukocytes are usually recruited as a consequence of an inflammatory response, the “leukolike” system might have the potential to be applied to the vast array of pathologies which involve inflammation. Moreover, the ability to genetically modify primary leukocytes with tumor targeting agents offers an additional powerful tool to optimize the “leukolike” system for a multitude of different cancer types.

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