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Proteomic Profiling of a Biomimetic Drug Delivery Platform

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Abstract

Current delivery platforms are typically designed for prolonged circulation that favors superior accumulation of the payload in the targeted tissue. The design of efficient surface modifications determines both a longer circulation time and targeting abilities of particles. The optimization of synthesis protocols to efficiently combine targeting molecules and elements that allow for an increased circulation time can be challenging and almost impossible when several functional elements are needed. On the other hand, in the last decade, the development of bioinspired technologies was proposed as a new approach with which to increase particle safety, biocompatibility and targeting, while maintaining the synthesis protocols simple and reproducible. Recently, we developed a new drug delivery system inspired by the biology of immune cells called leukolike vector (LLV) and formed by a nanoporous silicon core and a shell derived from the leukocyte cell membrane. The goal of this study is to investigate the protein content of the LLV. Here we report the proteomic profiling of the LLV and demonstrate that our approach can be used to modify the surface of synthetic particles with more than 150 leukocyte membrane-associated proteins that determine particle safety, circulation time and targeting abilities towards inflamed endothelium.

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CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

AUTHOR CONTRIBUTION

All authors have contributed to the manuscript and have given approval to the final version of the manuscript.

Keywords

Bio-mimetic camouflage; drug delivery; leukocyte; leukolike vector; membrane; nanotechnology; nanoparticles; proteomics

1. INTRODUCTION

The encapsulation of therapeutics in drug delivery systems was shown to improve bioavailability and the release kinetics of the therapeutics, and to provide superior protection from biological degradation in the blood and/or after cellular internalization. In addition, drug nano-encapsulation was shown to be a reliable approach to enable selective targeting of the tissues of interest, thus reducing the incidence of pharmacological side effects [1, 2]. Current delivery platforms are typically designed for prolonged circulation that favors superior accumulation of the payload in the targeted tissue by exploiting the enhanced permeation and retention effect of the inflamed vasculature, which is a common feature of many inflammatory diseases (e.g. cancer, autoimmune diseases) [3]. The design of efficient surface modifications determines both the prolonged circulation time of particles and their targeting abilities. Surface modifications based on polyethylene glycol (PEG) were shown to inhibit particle clearance by inhibiting electrostatic interactions between the carriers and biological surfaces, such as the protein matrix and cell membrane [4–6]. Unfortunately, this polymer was also recently shown to be immunogenic and, when applied in presence of targeting molecules (proteins, antibodies and peptides), it was found to affect their targeting efficiency [7]. The optimization of synthesis protocols to efficiently combine these two elements can be challenging and almost impossible when several kinds of functional elements are needed [8]. In the last decade, bioinspired technologies such as erythrocyte membrane-camouflaged polymeric carriers [9], macrophage-based nanoparticles [10], and virus-based nanoparticles [11, 12] were proposed as a new approach with which to increase particle safety, biocompatibility and targeting, while maintaining the synthesis protocols simple and reproducible. Our group recently introduced a new generation of injectable carriers namely the leukolike vectors (LLVs), obtained via a biomimetic camouflage of nanoporous silicon particles (NPS) coated with purified leukocyte membranes [13]. The leukocytes were chosen as cell membrane source due to their ability to freely circulate in the bloodstream, and to targeting the inflamed endothelium, as well as their easy isolation and ex-vivo expansion. Leukocyte membrane proteins play a significant role in various cellular processes such as cell-to-cell communication and cell signaling which determine their biocompatibility and ability in adhering and modulating endothelial barrier function upon surface contact [14]. We showed that the coating preserved its lipid and protein content and imparted synthetic nanoporous silica particles (NPS) with leukocyte-mimicking functions [13]. With a one-step surface modification based on electrostatic interaction between the negatively charged phospholipids and the positively charged silicon surface, LLVs were provided of superior circulation time and targeting towards tumor inflamed vasculature *in vitro* and *in vivo*, while allowing for increased vascular perfusion upon surface contact [13]. Herein we assess the potentialities of our protocol in modifying synthetic particles with multiple leukocyte molecules and receptors using an LC/MS-based proteomic approach and in identifying molecular pathways associated with leukocyte/endothelium interactions that

can play a significant role in determining LLV efficacy as a drug delivery platform designed for intravenous administration.

2. MATERIALS AND METHODS

2.1. Cell Culture and Membrane Purification

The J774 mouse macrophage cell line was purchased from the American Type Culture Collection (ATCC) and grown in Dulbecco's Modified Eagle Medium (DMEM) containing 10% (v/v) fetal bovine serum (FBS). Before membrane purification, 2.5×10^8 cells were centrifuged for 10 minutes at 500 x g and the pellet was solubilized as described elsewhere [13]. Cell membranes were isolated by ultracentrifugation using a discontinuous sucrose gradient consisting of 55-40-30% sucrose w/v in a normal saline solution and a Thermo Scientific™ SureSpin™ 630 rotor at 28,000 x g for 30 min at 4°C with acceleration set to 9 and deceleration set to 0. After ultracentrifugation, the membrane fraction was identified according to the expression of various leukocyte membrane markers [13]. The above-mentioned fraction at 30 to 40% interphase was then collected, washed twice with normal saline, dried overnight and stored at -20°C until use.

2.2. LLV Assembly and Physical Chemical Parameters Characterization

A Bradford assay (Bio-Rad, Hercules, CA) was employed to evaluate the protein concentration of the isolated plasma membranes. On the other hand, NPS were incubated with the membrane solution overnight at 4°C under rotation following the conditions previously established [13]. The size and the zeta potential values of the samples, i.e. NPS and LLV, were analyzed using the Multisizer 4 Coulter Counter (Beckman Coulter Inc., Miami, FL) and ZetaSizer Nano ZS (Malvern Instruments Ltd., Southborough, MA), respectively.

2.3. Scanning Electron Microscopy Analysis

For visual confirmation of the successful transfer of cellular membranes onto the NPS, we employed scanning electron microscopy. Briefly, samples were washed five times in normal saline solution at 300 x g for cells and 750 x g for particles. After the fifth wash, samples were resuspended in saline solution at a concentration of 5×10^6 / mL for cells or 1×10^7 / mL for particles. Subsequently, a 10 μ L sample was blotted onto an aluminum stub mount and allowed to dry in a desiccator. After drying, samples were sputter-coated with a 5 nm gold layer and imaged using an FEI Quanta 400 ESEM FEG instrument.

2.4. Sample Preparation and Trypsin Digestion for Pro-teomic Analysis

Samples were processed for proteomic analysis via peptide-level LC/MS^E analysis by in-solution trypsin digestion after reduction and alkylation of protein thiols; briefly as follows. Samples were solubilized with 0.5% RapiGest SF surfactant (Waters Corp, Milford, MA) in 50 mM ammonium bicarbonate solution (AMBIC) at pH 8.0, then treated with 5mM dithiothreitol (DTT) for 30 min at 60°C, and then with 15 mM iodoacetamide (IAA) for 30 min at room temperature in the dark. To de-lipidate samples, an extraction with methanol/chloroform was employed, following the experimental procedure previously described [15]. The protein pellet was re-suspended in 0.2% RapiGest SF and the protein concentration was

determined by Bradford (Bio-Rad) protein assay. Trypsin digestions were performed overnight at 37°C at an enzyme-to-substrate ratio of 1:50. Porcine Trypsin Sequencing Grade (Promega Corporation, Madison, WI) modified by reductive methylation to resist to proteolytic digestion was employed. Reactions were stopped by acidification of the digest solutions to 0.5% with trifluoroacetic acid. Peptides were recovered from the supernatant after centrifugation at 18,000 x g for 10 min to remove any particulate matter.

2.5. LC/MS^E Conditions

The LC/MS system consisted of a Waters Corp. NanoAcquity UPLC system coupled in-line with a Synapt HDMS (G1) mass spectrometer, via a nano-electrospray ion source. Tryptic peptides were chromatographically resolved over a C-18 column under reverse phase gradient conditions before being injected into the mass spectrometer in positive ion (ESI) mode. The LC system consisted of a 180 µm x 20 mm Symmetry C18 (5 µm particle) trapping column, and a 75 µm x 250 mm BEH130 C18 (1.7 µm particle) analytical column. Solvent A consisted of 0.1% formic acid in water, and solvent B consisted of 0.1% formic acid in acetonitrile. 0.5 µg aliquots of each trypsin digest sample were mixed with 100 fmol of a yeast alcohol dehydrogenase protein (yADH) tryptic digest that served as an internal standard for protein quantification normalization between sample sets, and was loaded onto the LC column. Peptide separation was accomplished by a 3–40% gradient of solvent B over 120 min at a column flow rate of 300 nl/min and a column temperature of 35°C. The mass spectrometer was operated in the data independent (parallel-ion fragmentation) MS^E mode [16–18] at a capillary voltage of 3 kV, with alternating low (6 V) and ramped high collision energies (15 V–45 V) at a scan rate of 1.2 sec per scan. An internal lock mass calibrant (Glufibrinopeptide B) was sampled every 30 secs from an alternate inlet in the source. All data were collected with the time of flight (TOF) detector set in the V-mode (resolution ~10,000) which had been externally calibrated. All LC/MS instrument control and data acquisition were accomplished using the MassLynx (v 4.1) software from Waters Corp. The analysis was performed in triplicate in randomized sequence.

2.6. Protein Identification

All raw MS data were lock-mass corrected after acquisition, and further processed for protein identification using the ProteinLynx Global Server (PLGS v2.4; Waters Corp) software. Chromatographic peak widths, TOF resolution, and precursor ion and fragment ion mass error tolerance levels (typically less than 5ppm and 15ppm respectively) were calculated automatically by the software. The Uniprot 2013_03 ('reviewed') complete mouse proteome database, with 16,614 entries to which the sequence for the yeast ADH internal protein standard was appended, was used as the target database. A reverse and randomized decoydatabase search strategy was employed by the PLGS software to estimate the false discovery rate (FDR) for protein identifications, and FDR limits were set at 1%. Peptide identifications were accepted with a minimum of 2 peptides and 7 fragment ions matched per protein, with a minimum of 4 fragment ions per peptide detected, with fixed modification of carbamidomethyl-cysteine, and oxidized-methionine as the only allowable variable modification. One trypsin miscleavage was also allowed in the database search strategy. All protein identifications were further filtered to retain only those protein IDs that remained above the 95% confidence interval. All quantification values for identified

proteins in the samples were normalized to the yADH internal protein standard (100 fmol yADH loaded on column per sample) and quantified for amounts loaded on column by the Identity^E [19] algorithm. All replicate data were included in the analyses.

2.7. Flow Cytometry Analysis

Two different samples were analyzed by flow cytometry to investigate the presence of selected proteins: NPS (2×10^6) coated with liposomes made by dipalmitoylphosphatidylcholine (DPPC), cholesterol (CHOL) and dioleoylphosphatidylcholine (DOPC) (5:2:3 molar ratio) representing our negative control; and NPS coated with J774 membranes (LLV, 2×10^6). Liposomes were assembled as previously described [20]. The samples were incubated with primary labeled monoclonal antibodies, FITC-conjugated anti-CD45 mAb, anti-MAC-1 mAb and anti LFA-1 (BD Biosystems Qume Drive San Jose, CA), in PBS with 1% BSA, pH 7.2, 1 h, under constant agitation in the dark. After incubation, the excess mAb was removed by dialysis using a membrane with a cutoff of 1000 kDa. The analysis was performed using a Becton Dickinson LSR Fortessa equipped with Diva software. Five thousand events were collected for each experiment. The results were the average of three independent experiments.

2.8. Bioinformatic Analysis

The whole protein data set (Table S1) identified was submitted to bioinformatics analysis using the DAVID Bioinformatics Resources 6.7 (<http://david.abcc.ncifcrf.gov/>) which helped to perform protein clustering based on molecular function and cellular component. Furthermore, to identify processes and pathways related to the identified proteins, we analyzed the group of proteins listed in Table S1 using the Ingenuity Pathway Analysis software (<http://www.ingenuity.com/>). IPA constructs a network of direct and indirect interactions between molecules based on data found in the literature. The parameters used to analyze data have been previously described [21–23].

3. RESULTS AND DISCUSSION

3.1. LLV Assembly and Physical and Chemical Characterization

Fresh leukocyte membranes were isolated from murine J774 macrophages using a purification method based on a discontinuous sucrose gradient [13]. NPS were previously APTES-modified to give the particles a positive surface charge and favor electrostatic interactions with the negatively charged phospholipids of the membrane, as previously reported [13]. The coating gave the LLVs a negative charge similar to that of the donor cells (J774: -19 mV; LLV: -16 mV) as shown in Fig. (1A). As expected, multisizer analysis showed that LLVs were slightly larger than NPS (Fig. 1B) thereby confirming the presence of a cellular membrane coating. Compared to the uncoated particle surface, characterized by a regular porosity, SEM images showed that the membrane homogeneously coated the NPS (Fig. 1C). LLVs were characterized by a typical surface roughness that, in comparison to that of the original J774 macrophages, appeared smoother and without the presence of membrane crinkles (Fig. 1C). This approach enabled efficient coating of synthetic particles irrespective of their size, shape or material (Fig. 2). For instance, APTES-modified spherical

plain PLGA or silica particles as well as cylindrical silicon nanoporous carriers can be easily cloaked with the cell membrane just by inducing electrostatic bounds.

3.2. Analysis of the LLV Whole Protein Content by LC/MS^E

We characterized the biological features of the LLV using proteomic analysis. After LLV assembly, the particles were extensively washed to remove any excess of cell membrane that did not adhere to the NPS surface. Then, the membranes were eluted from the LLV, and the proteins were identified by mass spectrometry. Finally, we analyzed the proteins using bioinformatics tools to identify biochemical pathways that the LLV could trigger when in contact with the vasculature surface (Scheme 1). Mass spectrometric analysis allowed for the identification of 334 different proteins that were transferred onto the LLV surface (Table S1). These proteins were classified based on the data currently available in the Gene Ontology Annotation and Uniprot databases. About 50% of the proteins were associated to the plasma membrane while the others belonged to various subcellular compartments such as the endoplasmic reticulum, mitochondria, ribosomes and nucleus (Table S1). This result was expected given the physiologic process of protein translation in the endoplasmic reticulum and shuffling by the Golgi that cycles them between organelles and the cell surface [24]. Furthermore, proteins initially classified as “organelle-specific” can reside in more than one subcellular compartment. In addition, many other studies of membrane purification reported a range of membrane-associated proteins between 37% and 81% of the total number of identified proteins [25–30]. In our study, the membrane-associated proteins transferred on the NPS’ surface were classified and clustered as: 32% integral or lipid-anchored, 37% cytoskeletal and/or junctional, 21% peripherally associated on inside, 7% externally bound-secreted, and 3% associated to membrane-bounded vesicles (Fig. 3A). The integral and lipidanchored membrane proteins were further characterized into functional categories according to the GO/UniProt annotation. Transport, signaling and immunity (Fig. 3B) were the three main functions associated with the identified proteins.

3.3. Validation of the Expression of Leukocytes Markers on LLV

We next verified the presence and correct orientation on the LLV surface of three leukocyte markers involved in functions of our interest. To this aim, we performed a flow cytometry analysis directly on the LLV. In this experiment, the NPS coated with liposomes, i.e. phospholipid membranes without any protein were used as a negative control. The samples were analyzed for the presence of two receptors involved in the adhesion of leukocytes to endothelial cells, i.e. macrophage-1 antigen (MAC-1) and lymphocyte function-associated antigen 1 (LFA-1) [31, 32], and of a leukocyte common antigen (CD45), which is expressed on all leukocytes and is involved in the establishment of self-tolerance proprieties in these cells [33, 34] (Fig. 4). The antibodies used to perform this analysis were specifically selected to interact with the extracellular portion of these membrane receptors to evaluate their orientation on the LLV surface. The LLV showed strong signals correlated with all three molecules, while the NPS used as a negative control, did not emit any signal.

3.4. Functional Annotation of the LLV Proteins

A functional analysis of the proteins identified on the LLV surface was performed using the Ingenuity Pathway Analysis (IPA) software that enables the coupling of proteins with the

respective molecular pathway (Fig. 5). The p -value was calculated using the right-tailed Fisher's exact test. Pathways with a p -value < 0.05 were considered to be statistically significant. The analysis of the canonical pathways provided by the IPA program confirmed the enrichment of membrane proteins linked to canonical leukocyte pathways including the leukocyte extravasation pathway (Fig. 5). We also evaluated the whole protein data set by DAVID and among the KEGG pathways matching our proteins, the leukocyte transendothelial migration pathway was significant (p -value: 5.7×10^{-6}) (Fig. 6, red stars are proteins identified on the LLV surface). Transendothelial migration enables the leukocyte to specifically interact and adhere to the inflamed endothelium as well as to migrate and infiltrate the sub-endothelial space [35]. Both IPA and DAVID revealed that this is one of the highest scoring pathways associated with the proteins found in the LLV, which confirms that our approach was capable of transferring multiple leukocyte markers involved in the interaction with the inflamed endothelium and that the transfer of leukocytes membranes on synthetic nanoparticles may equip them with cell-like functions.

CONCLUSION

The ideal drug delivery system should be able to circulate in the blood stream, target the diseased tissues and locally release its payload. Typical synthetic approaches can be inefficient in providing multiple skills and functions to drug delivery platforms, and bio-inspired approaches represent an emerging alternative approach to reach this goal. In this paper, we report a proteomic study that demonstrates the potentialities of our previously described method [13] to efficiently formulate a coating enriched with multiple leukocytes-membrane proteins with the aim of providing synthetic drug delivery systems endowed with cell-like functions. We estimated that about 50% of the proteins identified in the LLV are membrane-associated proteins and also demonstrated that most of them are involved in biological processes like transport, adhesion and signaling. Our approach enabled the transfer of more than 150 proteins associated with leukocyte membranes and at least a portion of them maintained the correct orientation. These studies confirm the potential of this approach to easily produce a bio-inspired drug delivery system and provide a general strategy that can be applied to different kinds of material and carriers. Bioinformatics analysis revealed that one of the most significant pathways associated with the proteins transferred to the silicon particles is the leukocyte transendothelial migration pathway, which confirms the advantages of using this method to impart onto synthetic particles, a natural ability to interact with inflamed endothelium. On the other hand, additional investigations are needed to optimize the cell membrane purification process due to the presence of proteins belonging to other cellular compartments.

These findings provide the proof of concept and showcase how the cell membrane can be manipulated to efficiently design the surface of delivery platforms. This procedure opens new opportunities based on pure or chimerical coatings purified from different cell phenotypes (i.e. cancer cells, organ-derived cells, red blood cells, platelets, leukocytes) to potentially tune the pharmacokinetic and toxicological profile of carriers. Lastly, a more deeper and integrated investigation of the proteomic, lipidomic and glycosidic profile of the coating could help to understand better biologic processes like cell-cell interaction and cell-signaling.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

CHOL	Cholesterol
DPPC	Dipalmitoylphosphatidylcholine
DOPC	Dioleoylphosphatidylcholine
LC	Liquid chromatography
LLV	Leukolike vector
MS^E	Alternating acquisition of low and high collision energy mass spectrometry
NPS	Nanoporous silicon particle
PBS	Phosphate buffer saline

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Fig. (1). Nanoparticles characterization

A) Zeta potential analysis of oxidized and APTES-modified NPS; APTES modification induces a switch in the surface charge from negative to positive. The LLV zeta potential seems to perfectly match the J774 cell surface charge. B) Multisizer analysis of NPS and LLV showing a slight increase in particle size after the coating with proteolipid membranes. C) SEM images of (from left to right) oxidized NPS, J774 cell used as a membrane source and LLV coated with leukocyte-derived membrane. Scale bar, 1 μm .

Fig. (2). SEM images of spherical PLGA and silica particles, and cylindrical silicon particles (top row) coated with purified leukocyte membranes reveal uniform distribution of membrane coating on the particle surface (bottom row).

Fig. (3). Classification of proteins identified on LLV

Pie charts of the identified proteins classified according to UniProt/GO information and manually searching of the literature; A) Classification of subclasses of plasma membrane-associated proteins; B) Functional characterization of the plasma membrane-associated proteins.

Fig. (4). Flow cytometry analysis of selected proteins

The presence of LFA-1, MAC-1 and CD45 on the LLV surface was confirmed by FACS analysis. The results showed a strong signal associated to all these proteins on the LLV while no signal is present on the NPS thus confirming the presence and the proper orientation of these proteins on the LLV surface.

Fig. (5). Ingenuity Pathway Analysis (IPA)

The identified membrane proteins were analyzed using the IPA software. A) Selected canonical pathways associated by IPA to the whole data set of identified proteins. The yellow threshold indicated a 95% confidence while the line represents the ratio of the number of genes in a given pathway which meets the cutoff criteria divided by the total number of genes which constitute the pathway. B) On the basis of data from the literature, IPA constructs a network of direct (solid line) and indirect (dotted line) interactions between molecules. Proteins in grey were identified on LLV, while proteins in white were not

identified in our analysis but were inserted automatically by the software because they are biologically connected to our proteins. The blue lines indicate proteins identified on LLVs that are involved in “Leukocyte Extravasation Signaling”.

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Fig. (6). Leukocyte Transendothelial Migration

The stars represent proteins identified on LLV matched on the KEGG pathway “Leukocyte Transendothelial Migration” using DAVID software.

Scheme 1. Schematic of experimental workflow

The following steps were performed: membrane isolation from leukocytes, coating of NPS with the extracted membranes, washing in PBS, elution of the membrane proteo-lipid component from the LLV, sample preparation for mass spectrometry analysis, protein identification and data mining.