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Size and charge of nanoparticles following incubation with human plasma of healthy and pancreatic cancer patients

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ABSTRACT

When nanoparticles (NPs) enter a biological environment, proteins bind to their surface forming a protein coating, which alters NP features giving it a biological identity, which controls its physiological response. The NP biological identity (size, charge and aggregation state) does strictly correlate with its physicochemical properties and the nature of the biological environment. While the former relationship has been extensively investigated, whether and how alterations in the physiological environment affect the biological identity of the NPs remains unclear. In this work we enrolled healthy and histologically proven pancreatic cancer patients. A statistically significant reduction in the level of clinically relevant proteins in cancer patients occurred. Positively and negatively charged lipid nanoparticles with two different surface chemistries (plain and PEGylated) were incubated with human plasma from both groups and characterized thoroughly by dynamic light scattering and zeta potential measurements. Only when plain positively charged NPs were tested, significant difference in zeta-potential between healthy and pancreatic cancer groups was found. This result implies that pooling human plasma from healthy volunteers might lead to a bias and thus unpredictable consequences in regard to previously optimized targeting profile.

1. Introduction

In the last decade, an outburst in nanotechnology has revolutionized the field of medicine opening intriguing new avenues for improving both therapeutics and diagnostic [1]. Nanoscale materials possess unusual physical, chemical and biological properties leading to unprecedented opportunities such as increased solubility and in vivo stability, minimized side effects and superior therapeutic index. The hardest challenge occurs when nanomaterials are injected into biological environments, such as blood [2–5]. Due to their high surface free energy, nanomaterials adsorb biomolecules, especially proteins, forming a biological coating referred to as the protein corona [6,7]. As a consequence of protein binding the nanomaterial acquire a 'biological identity' (size, charge and aggregation state) that is rather different from its synthetic one [8]. What is clear to date is that this new identity is the main factor controlling biodistribution, therapeutic effect and nanomaterials nanotoxicity in the body. The bio-identity of a given nanomaterial in vivo is given by the size, surface charge and composition of the protein corona [9]. While size and surface charge of NP–protein complexes control the plethora of not specific interactions with blood components and target cells, the composition of the protein coating [8] can interfere with active targeting by introducing competing biological signals [10]. Dawson et al. have been the first to propose that what target cells really "see" is not the bare nanomaterial as originally synthesized, but rather its "protein corona" [11–13]. More in detail, cell–nanomaterial interactions could be controlled by the identity, arrangement and residence time of the proteins at the particle surface. The adsorbed protein layer would incorporate proteins engaged from the blood that could let the nanoparticle to interact with specific receptors expressed on the plasma membrane of target cells. This 'protein corona effect' [14] is a formidable challenge that could lead to a complete renewal of the current strategies of targeted drug delivery. It is clear that the 'protein corona effect' requires a very accurate identification and quantification of plasma proteins to be exploited. This is guaranteed by recent progresses in proteomics, particularly in terms of mass accuracy, speed and resolution [15]. Taking advantage of these challenging opportunities some authors have recently exploited the "protein corona" to target specific cell types in a controlled

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manner [16–18]. In a very recent study, some of us have shown that cationic liposomes/DNA complexes, which spontaneously become coated with vitronectin upon interaction with human plasma (HP), promote efficient uptake in cancer cells expressing high levels of the vitronectin $\alpha_v\beta_3$ integrin receptor [19]. However, these pioneering studies, failed to recognize that concentration of several proteins in cancer patients could be different from its counterpart in healthy controls. This implies that the nanomaterials' bio-identity after interaction with human plasma of cancer patients may differ from that acquired after interaction with human plasma collected from healthy volunteers. This suggestion is supported by recent findings showing that incubating NPs with human plasma from subjects with different diseases and medical conditions results in protein coronas of different composition [20]. This is a hot topic for targeted drug delivery because the possibility to target cancer cells by the protein corona depends on the proteins directly engaged from the blood [10,19,21]. Indeed the development of a targeted drug using blood samples from healthy subjects, might lead to a bias and thus unpredictable consequences in regard to previously optimized targeting profile. In this regard, we believe that future developments in the field of nanomedicine will be based on the concept of "personalized protein corona" [20].

To shed light on this key issue, herein we used liposomes, which are currently tested for numerous applications in nanobiomedicine [22] and are already used in cancer treatment. In search of general principles, we decided to use liposomes of various charge and surface modification. Lipid particles were incubated with human plasma from both healthy volunteers and pancreatic cancer patients and characterized thoroughly by dynamic light scattering (DLS) and zeta-potential measurements.

We decided to investigate pancreatic cancer for it represents a leading cause of cancer related mortality and a very aggressive tumor. Despite the last decade's efforts, the overall 5-year survival amount to less than 5%. Only early diagnosis (stage I) and pancreatic cancer radical surgical resection allow survival rates up to 32.8% [23]. Unfortunately, to date only 7% of pancreatic cancers are diagnosed at stage I and less than 15–20% can be resected because of the high rate of local or distant progression of the disease. Thus, in un-resectable patients, chemotherapy and radiotherapy can only achieve minimal benefits because of pancreatic neoplastic cells resistant behavior to drugs and radiations [24]. On this basis, scientific community agrees on the needing of new tools able to improve the outcome of this challenging disease.

2. Materials and methods

2.1. Demographic characteristics

The present study has been approved by the Ethical Committee of the University Campus Bio-Medico di Roma. Between March 2013 and July 2013, we collected blood samples from 11 consecutive histologically proven pancreatic cancer patients and from a group of 13 consecutive patients affected by surgical benign diseases that met the criteria reported in Table S1. In the following text, the latter patients will be referred to as 'healthy patients' and their group as 'healthy group'. In our study, the term healthy will mean that patients meeting criteria listed in Table S1 are not affected by cancer. Data regarding medical history and clinical-instrumental work-up have been assessed for each patient; particularly, total protein, serum albumin and serum protein electrophoresis have been considered in both groups. Total protein and albumin were dosed on Dimension Vista (Siemens) system. A colorimetric method (endpoint polychromatic) with bromocresol purple was routinely used for albumin [25]. Proteins have been measured by bichromatic endpoint using modified Biuret. Obtained values are expressed in g/dl for both albumin and total protein. Electrophoresis has been performed on Capillarys Sebia electrophoresis system for half an hour (zonal electrophoresis in liquid phase). It is a semi-quantitative analysis and values of various protein fractions (α_1 (e.g. anti-trypsin), α_2 globulin (e.g. α_2-macroglobulin, aptoglobine, ceruloplasmine), β_1 globulin (e.g. transferrin), β_2 globulin (e.g. β_2-microglobulin), γ globulin (e.g. immunoglobulin)) are expressed as percentage intervals.

2.2. Blood collection and plasma preparation

Venous blood samples have been collected using the usual puncture technique and stored in TM BD P100 Blood Collection System tubes containing K2EDTA and protease inhibitor (Franklin Lakes, NJ, USA 7). The samples have been centrifuged at 1800 rpm for 10 min and plasma was stored in LoBind tubes (Eppendorf) at −80 °C. When used, aliquots were thawed at 4 °C and then left to warm at room temperature.

2.3. Liposomes preparation

1,2-Dioleoyl-3-trimethylammonium-propane (DOTAP), 1,2-dioleoyl-*sn*-glycero-3-phospho-rac-(1′-glycerol) (DOPG), dioleoylphosphocholine (DOPC), dioleoylphosphatidylethanolamine (DOPE), DOPE/polyethyleneglycol 2k (DOPE/PEG2k) and cholesterol (Chol) were purchased from Avanti Polar Lipids (Alabaster, AL) and used without further purification. DOTAP-Cholesterol (0.5:0.5 molar ratio) cationic particles (CPs) and DOPG-DOPC-DOPE (0.5:0.25:0.25 molar ratio) anionic particles (APs) were prepared following standard procedures [26]. Briefly, each lipid mixture, at a molar ratio of neutral lipid in the bilayer Φ = (neutral lipid/total lipid) (mol/mol) = 0.5), was dissolved in chloroform and the solvent was evaporated under a stream of nitrogen and then under a vacuum for 12 h. The obtained lipid films were hydrated with the appropriate amount of deionized water to achieve the desired final concentration of 1 mg/mL. Lipid films were hydrated (final lipid concentration 1 mg/mL) with ultrapure water and stored at 4 °C. In PEGylayed CPs, 10 mol% of cholesterol was substituted with DOPE/PEG2k. 10 mol% of PEGylated lipids is pretty large considering that available lipid formulations usually contain between 1 and 5 mol% PE-PEG2000 relative to the total lipid content. However, we have recently shown that liposome formulations with 10% of PE-PEG2000 exhibit extraordinary cellular uptake in cancer cells [27]. Thus, we chose to employ such density of PE-PEG2000 in view of a forthcoming in vivo experimentation. As a result, lipid composition of PEGylated CPs was: DOTAP-cholesterol-DOPE/PEG (0.5:0.4:0.1 molar ratio). For preparing PEGylated APs 10 mol% of DOPE was substituted with DOPE/PEG2k with the result that final composition was: DOPG-DOPC-DOPE-DOPE/PEG (0.5:0.25:0.15:0.1 molar ratio).

2.4. Size and zeta-potential experiments

Landmark studies [6,8] have shown that the aggregation state of nanoparticles, thickness and composition of the protein corona can change with plasma concentration. However, saturation occurs at around 40–80% plasma concentration depending on the NP's nature [6]. According to this indication, we have previously investigated the effect of plasma concentration on the composition of the liposome–protein corona [28]. We found that saturation occurs at 50%, thus in this study we used 50% plasma concentration (i.e. 1:1 liposome:HP volume ratio). For size and zeta-potential experiments, lipid NP–HP complexes were prepared by incubating 10 μL of each lipid dispersion, pre-diluted with 490 μL of ultrapure water, with 10 μL of HP pre-diluted with 490 μL of ultrapure water. Dilution did not change the pH of HP (pH = 7.45 ± 0.03). After