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Protein corona as a proteome fingerprint: The example of hidden biomarkers for cow mastitis

Giovanni Miotto[a,b], Massimiliano Magro[c,d], Milo Terzo[c], Mattia Zaccarin[a,b], Laura Da Dalt[c], Emanuela Bonaiuto[c], Davide Baratella[c], Gianfranco Gabai[c], Fabio Vianello[c,d,*]

[a] Department of Molecular Medicine, University of Padua, Italy
[b] Proteomics Facility, Azienda Ospedaliera di Padova, University of Padua, Italy
[c] Department of Comparative Biomedicine and Food Science, University of Padua, Italy
[d] Regional Centre of Advanced Technologies and Materials, Department of Physical Chemistry and Experimental Physics, Faculty of Science, Palacky University, 17. Listopadu 1192/12, 771 46 Olomouc, Czech Republic

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ABSTRACT

Proteome modifications in a biological fluid can potentially indicate the occurrence of pathologies, even if the identification of a proteome fingerprint correlated to a specific disease represents a very difficult task. When a nanomaterial is introduced into a biological fluid, macromolecules compete to form a protein corona on the nanoparticle surface, and depending on the specific proteome, different patterns of proteins will form the final protein corona shell depending on their affinity for the nanoparticle surface. Novel surface active maghemite nanoparticles (SAMNs) display a remarkable selectivity toward protein corona formation, and they are able to concentrate proteins and peptides presenting high affinities for their surface even if they are present in very low amounts. Thus, SAMNs may confer visibility to hidden biomarkers correlated to the occurrence of a pathology. In the present report, SAMNs were introduced into milk samples from healthy cows and from animals affected by mastitis, and the selectively bound protein corona shell was easily analyzed and quantified by gel electrophoresis and characterized by mass spectrometry. Upon incubation in mastitic milk, SAMNs were able to selectively bind α_{s2}-casein fragments containing the FALPQYLK sequence, as part of the larger casocidin-1 peptide with strong antibacterial activity, which were not present in healthy samples. Thus, SAMNs can be used as a future candidate for the rapid diagnosis of mastitis in bovine milk. The present report proposes protein competition for SAMN protein corona formation as a means of mirroring proteome modifications. Thus, the selected protein shell on the nanoparticles results in a fingerprint of the specific pathology.

1. Introduction

Bovine milk is a major source of animal proteins for human nutrition. European Union legislation underlines that milk selected for human nutrition must originate from healthy animals [24]. The quality of milk can be altered by several factors, such as nutrition interventions, the stage of lactation and the animal's age. However, mammary infections (mastitis) are the most serious threats to the health of the cow and milk quality, and they can cause severe economic losses in the dairy industry [32].

When pathogens elude the defenses of the mammary gland, they trigger an innate immune response, which consists of attracting circulating polymorphonuclear neutrophils (PMN) and other leukocytes to the site of infection. As a consequence, the somatic cell count (SCC) in milk increases. The SCC is the most generally used indicator of udder health, and milk quality payments are based on the SCC [32]. To differentiate between healthy and unhealthy mammary glands, SCC thresholds of 100,000 cells mL^{-1} [26] or 200,000 cells mL^{-1} [25] are generally used, although inflammatory processes can be observed in apparently healthy mammary glands with SCCs lower than 100,000 cells mL^{-1} [26].

Both bacteria and PMNs release oxidants, hydrogen peroxide and hypochlorous acid [3], and proteases, such as cathepsins B and

* Corresponding author at: Department of Comparative Biomedicine and Food Science, University of Padua, Agripolis—Viale dell'Università 16, Legnaro, 35020 (PD), Italy. Fax: +39 049 8272973.
E-mail address: fabio.vianello@unipd.it (F. Vianello).

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D and elastase [10,8], which can damage the mammary cells and milk components, milk proteins in particular [32]. The resulting milk proteome and peptidome show peculiar differences in comparison to those from healthy animals [6,18].

Ideally, proteomics, as a large-scale study of proteins, could be employed in diagnostics by individuating specific proteome variations correlated with diseases. Notwithstanding, the complexity of the proteome does not allow easy interpretation of its changes. On the other hand, protein corona formation on a specific nanoparticle surface is the expression of a particular proteome, where proteins displaying the highest affinity for the nanoparticle surface can be discriminated. In this context, proteolytic events correlated to diseases can change the proteome scenario as well as the relative affinities for nanomaterial surfaces ruling the competition for the formation of protein coronas. That is, proteolysis may change the chemical nature of a large set of proteins thereby drastically modifying the competition for a nanoparticle surface. Thus, the binding of specific protein fragments on the surface of a nanoparticle can indicate a disease, and the proteolysis products can be exploited as markers of pathology. Furthermore, protein corona formation on nanomaterials depends on the chemical nature, size and shape of the employed nanomaterial [4].

Magnetic nanoparticles have already demonstrated their potential in biomedical applications [33,9], as these nanostructures can serve for magnetic separations [30,34], drug delivery systems [20] or to generate heat by exposure to alternating electromagnetic fields [11], thus increasing the temperature of tumor tissues and destroying pathological cells. Moreover, their use in magnetic resonance imaging as contrast agents is common [27,7] due to their unique magnetic properties and biocompatibility [12].

An essential prerequisite for the implementation of bionanotechnological applications is to obtain nanoparticles with a hydrophilic surface able to maintain colloidal stability under physiological conditions [28]. However, obtaining monodispersed colloidal suspensions of nanoparticles in aqueous media is a challenging task due to the complexity of controlling the nucleation and growth processes in water. Recently, we developed a new simple wet method to synthesize naked superparamagnetic nanoparticles consisting of stoichiometric maghemite (γ-Fe_2O_3) with dimensions of approximately 10 nm, and monodispersed preparations in aqueous media could be obtained [13,14]. This new category of magnetic nanoparticles was called “surface active maghemite nanoparticles” (SAMNs) due to their specific surface chemical behavior without any superficial modification or coating derivatization. They are freely stable in water for several months as colloidal suspensions, present a high average magnetic moment and can be easily derivatized to immobilize specific organic molecules in solution. SAMNs have already been functionalized with proteins and DNA by simple incubation in aqueous solution [15,31,17].

In this novel experimental approach, we investigated the specific binding behaviors of whey proteins and protein fragments on SAMNs in milk samples characterized by low and high SCC values. The outcomes of the present work may lead to the identification of novel biomarkers to study the pathophysiology of bovine mastitis, mammary cell turn-over and tissue repair. In addition, the selected protein shell on nanoparticles can result in a fingerprint of a specific pathology, and the results might be exploited to develop improved diagnostic tools, in particular for mastitis.

2. Experimental

2.1. Materials

Chemicals were purchased at the highest commercially available purity and were used without further treatment. Iron(III) chloride hexahydrate (97%), sodium borohydride ($NaBH_4$), and ammonium hydroxide solution (35% in water) were obtained from Sigma–Aldrich (Italy).

Buffers were prepared according to standard laboratory procedures using Milli-Q reagent grade water (Merck Millipore, Billerica, MA, USA).

2.2. Instrumentation

Optical spectroscopy measurements were performed on a Cary 50 spectrophotometer (Varian Inc., Palo Alto, CA, USA) in quartz cuvettes (1 cm p.l.). FT-IR spectra were acquired using a Thermo Nicolet Nexus 670 instrument. Nanoparticle samples were lyophilized, homogenized with KBr powder, and pelleted by an 8.0 ton hydraulic press. Microscopic characterizations of SAMNs nano-bio-conjugates were performed by transmission electron microscopy (TEM) using a FEI Tecnai 12 microscope operating at 120 kV with a point-to-point resolution of 1.9 Å.

2.3. Synthesis of Iron Oxide Nanoparticles

A typical nanoparticle synthesis was previously described [13,14] and can be summarized as follows: $FeCl_3{\cdot}6H_2O$ (10.0 g, 37 mmol) was dissolved in MilliQ grade water (800 mL) under vigorous stirring at room temperature. A $NaBH_4$ solution (2 g, 53 mmol) in ammonia (3.5%, 100 mL) was then quickly added to the mixture. Soon after the reduction reaction occurred, the temperature of the system was increased to 100 °C and kept constant for 2 h. The material was cooled at room temperature and aged in water, as prepared, for another 12 h. This product was separated by imposition of an external magnet and washed several times with water. This material can be transformed into a red brown powder (final synthetic product) by drying and curing at 400 °C for 2 h. The resulting nanopowder showed a magnetic response upon exposure to a magnetic field. The final mass of product was 2.0 g (12.5 mmol) of Fe_2O_3, and a yield of 68% was calculated.

The nanoparticulated resulting material was characterized by zero field and in field (5T) Mossbauer spectroscopy, FTIR spectroscopy, high resolution transmission electron microscopy, XRPD, and magnetization measurements [14]. The material consisted of stoichiometric maghemite (γ-Fe_2O_3) with a mean diameter of 11 ± 2 nm, which can lead to the formation, upon sonication in water (Falc Instruments, Italy, mod. LBS1, 50–60 Hz, 500 W), of a stable colloidal suspension without any organic or inorganic coverage. The surfaces of these bare maghemite nanoparticles show peculiar binding properties and can be reversibly derivatized with selected organic molecules [16,17]. We called these bare nanoparticles surface active maghemite nanoparticles (SAMNs).

2.4. Milk samples and whey preparation

Milk samples were collected from dairy cows with no signs of mammary infection showing low somatic cell counts ($30{,}000 < SCC < 100{,}000$ cells mL^{-1}; $n = 4$) and from dairy cows with high somatic cell counts ($400{,}000 > SCC > 900{,}000$ cells mL^{-1}; $n = 4$), which is indicative of mammary inflammation. Somatic cells were counted by the official laboratory of the local farmers' association (ARAV) using a Fossomatic apparatus (Foss, Hillerød, Denmark).

Milk samples were centrifuged ($5000 \times g$, 15 min, 4 °C) to remove the fat and obtain skim milk. Then, whey was obtained from skim milk by removing casein micelles by ultra-centrifugation ($25{,}000 \times g$, 30 min, 4 °C).