Blood and Synovial Microparticles as Revealed by Atomic Force and Scanning Electron Microscope

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Abstract: Microvesicles which are pinched off the cell membrane can be considered extracellular organelles which mediate interaction between distal cells. They were suggested to play an important role in many diseases including autoimmune disorders, however, standard methods for their assessment have not yet been decided upon while their clinical relevance and the underlying mechanisms are yet unclear. We present a pilot study results involving atomic force microscope (AFM) and scanning electron microscope (SEM) images of the material isolated from peripheral blood of healthy donors and from synovial fluid of patients with psoriatic arthritis and rheumatoid arthritis, which is expected to contain microvesicles. Micrographs reveal in the samples isolated from blood the presence of globular and tubular structures which are most probably microvesicles while the identity of grain-like structures isolated from synovial fluid remains obscure. To the best of our knowledge the AFM and SEM images of the material isolated from synovial fluid are presented for the first time.

Keywords: Microvesicles, microparticles, shape, coagulation, antiphospholipid syndrome, thrombosis, cancer.

INTRODUCTION

Microvesicles [1-4] are formed in the process of budding. In the final stage of this process buds are pinched off the mother membrane and become free to move in the surrounding fluid. They are present in all body fluids and travel with lymph and blood to distal cells. Microvesicles can fuse with distal cells and thereby convey matter and information to these cells. All cells can shed microvesicles, therefore in principle there is no local event in the body.

Microvesicles were found to play an important role in different diseases such as autoimmune diseases [5-8], cardiovascular disorders [9, 10] and cancer [11-17]. Thromboembolic events, which are also manifestations of some types of cancer [18] and some autoimmune diseases [19], were associated with presence of microvesicles [20, 21]. Studies of the origin of microvesicles isolated from peripheral blood have shown that the largest pool comes from platelets (around 80%), erythrocytes contribute around 10%, while the remaining 10% originates from other cells (T-helper cells, T-supressor cells, monocytes, B-lymphocytes, granulocytes and endothelial cells) [22]. Neoplastic cells are prone to shed microvesicles, however, the significant increase of the number of microvesicles found in peripheral blood of patients with certain types of cancer originates from platelets which can be activated by microvesicles from neoplastic cells [15].

Microvesicles and mechanisms underlaying their formation are yet poorly understood, however, it is indicated that they are potentially interesting in diagnosis and treatment of different diseases. For that, a standard protocol should be elaborated and connected to the clinical status of populations as well as of individual persons. For example, analysis of the number and composition of microvesicles could be of help in defining the antiphospholipid syndrome, especially since recognizing the presence of particular antibodies does not yet give a decisive answer. For example, the abundance of endothelial and platelet microvesicles was indicated to correlate more specifically with thrombotic manifestations of antiphospholipid syndrome than antiphospholipid antibody levels alone and might thus provide an additional information for defining the disease [7, 8, 23]. Also detection of microvesicles could be used in conjunction with perceived gold-standards (tissue biopsy, angiography) for the diagnosis of active vasculitis [24] and for assessing the activity of rheumatoid arthritis [25].

It is therefore of interest to better understand microvesiculation and develop methods for assessment of microvesicles for the needs of scientific studies and of clinical practice. Different protocols for isolation of microvesicles can be found in the literature [22, 26-31], usually consisting of centrifugation and washing of the

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sample. To our knowledge the contents of the isolated material obtained by different protocols has not yet been visualized. Here we present the atomic force and scanning electron microscope images of microvesicle-rich plasma and of material isolated from synovial fluid. It is our aim to recognize microvesicles in the material, and determine their morphology and distribution with respect to size. This would enease also interpretation of measurements obtained by other techniques such as flow cytometry.

We present images of the material isolated from peripheral blood of three healthy donors and of the material isolated from synovial fluid of inflammed knee joints of a patient with psoriatic arthritis and a patient with rheumatoid arthritis.

2. MATERIAL AND METHODS

2.1. Donors

Donors of blood samples were two authors (subject H1: female, 49 years, subject H3: male, 26 years), and a volunteer (subject H2: male, 48 years), all with no record of a disease. Donors of synovial fluid samples were a patient with psoriatic arthritis (subject P1; female, 50 years) and a patient with rheumatoid arthitis (subject P2: female, 50 years). Written informed consent was obtained from donors. The research program including the studies on microparticles was approved by National Ethics Committee.

2.2. Collection of Blood and Synovial Fluid Samples

After 12 hour fasting, venous blood was collected from donors into 2.7 ml vacutubes (BD Vacutainers), containing 0.109 M trisodium citrate. Synovial fluid was collected by the joint aspiration from actively inflammed knee joints of patients with psoriatic/rheumatoid arthritis into 2.7 ml vacutubes (BC Vacutainer), containing 300 μL of 0.109 M trisodium citrate. Immediately after the collection of synovial fluid a further 300 μL of 0.109 M trisodium citrate was added to the synovial fluid to prevent clotting as described in [32]. Cells were removed from plasma/synovial fluid by centrifugation for 20 minutes at 1550 x g and 20°C. 225μL of microvesicle-rich plasma were used for atomic force microscope imaging.

2.3. Preparation of Microvesicle-Rich Plasma/Synovial Fluid and Isolation of Microvesicles

250μL aliquotes of microvesicle-rich plasma/synovial fluid were used for the isolation of microvesicles. Plasma/synovial fluid was centrifuged for 30 minutes at 17.570 x g and 20°C. After the centrifugation, 225 μL of microvesicle-poor plasma/synovial fluid supernatant were removed. According to the protocol described by Diamant *et al*. [22], 25 μL of the microvesicle pellet with the remaining plasma/synovial fluid were resuspended in 225 μL of phosphate buffered saline (PBS; 137mM NaCl, 2.7mM KCl, 7.8 mM Na_2HPO_4 x $2H_2O$, 1.5mM KH_2PO_4, pH 7.4), containing 10.9 mM trisodium citrate. After the second centrifugation (30 minutes, 17.570 x g and 20°C) supernatant (225 μL) was removed. For flow cytometric analysis, isolated plasma/synovial microvesicles were resuspended in 75 μL of PBS/citrate. For scanning electron microscopy (SEM) analysis, isolated plasma/synovial microvesicles were resuspended in the remaining ~ 25 μL of the supernatant. Aliquotes of 25 μL of resuspended microvesicles were collected into a single 1.5 mL Eppedorf tube and PBS/citrate was added to the final volume of 250 μL. The sample was centrifuged again (60 minutes, 20.000 x g, 20°C). Peleted microvesicles were subsequently prepared for SEM analysis as described in Section 2.5.

2.4. Atomic Force Microscopy (AFM)

Microvesicle-rich plasma/isolated synovial microvesicles were fixed to cleaved mica, with atomically smooth surface and incubated on mica for 15 min. After that the samples were rinsed with buffer solution and fixed with 1% formaldehyde in order to prevent changes in morphology during AFM analysis. Measurements with AFM (Solver PRO, NT-MDT, Russia) were done in the tapping mode at room temperature in air. The samples were scanned with standard Si cantilever (MikroMash) with force constant of 0.2 N/m, tip radius less then 10 nm and operating at resonance frequency 184 kHz.

In the phase mode imaging, the phase shift of the oscillating cantilever relative to the driving signal is measured. This phase shift can be correlated with specific material properties which depend on the tip-sample interaction. Therefore the phase shift can be used to differentiate areas on a sample with different properties, such as friction, adhesion, and viscoelasticity.

2.5. Scanning Electron Microscopy (SEM)

Microvesicles were suspension-fixed in 1% glutaraldehyde in the PBS/citrate buffer for 60 minutes at 22°C, post-fixed in 1% OsO4 in 0.9% NaCl for 60 minutes at 22°C, dehydrated in a graded series of acetone/water (50–100%, v/v), critical-point dried, gold-sputtered, and finally examined in a Cambridge Instruments S360 microscope.

2.6. Flow Cytometry

Flow cytometric data acquisition and analysis were performed on a flow cytometer (Altra flow cytometer, Beckman Coulter Inc., Fullerton, CA) with a 488-nm water-cooled laser.

Flow-Count fluorospheres of a known concentration (1 μm diameter, 9.7 x 10^5/mL) from Beckmann-Coulter (Fullerton, CA) were added to the sample to determine the percentage of the measured events. The presence of microvesicles and other particles smaller than 1 μm was determined by forward-scatter/side-scatter (FS/SS) dot plot. A minimum of 1 x 10^4 events was collected for each analysis. For the analysis, 50 μL of the sample containing microvesicles and 10 μL of fluorospheres were diluted in 150 μL of PBS-citrate. Beckman Coulter software EXPO32 was used for presentation of results.

3. RESULTS

Fig. (**1**) shows the atomic force micrograph of a sample obtained from microvesicle-rich blood plasma of a healthy donor H1. The height images (**a**, **b**, **d**) and the phase image (**c**) are presented. The globular structures, which differ in elasticity from the surroundings and protrude out of the surface, presumably correspond to microvesicles ranging from 100 to 300 nm in width and from 30 to 60 nm in height.

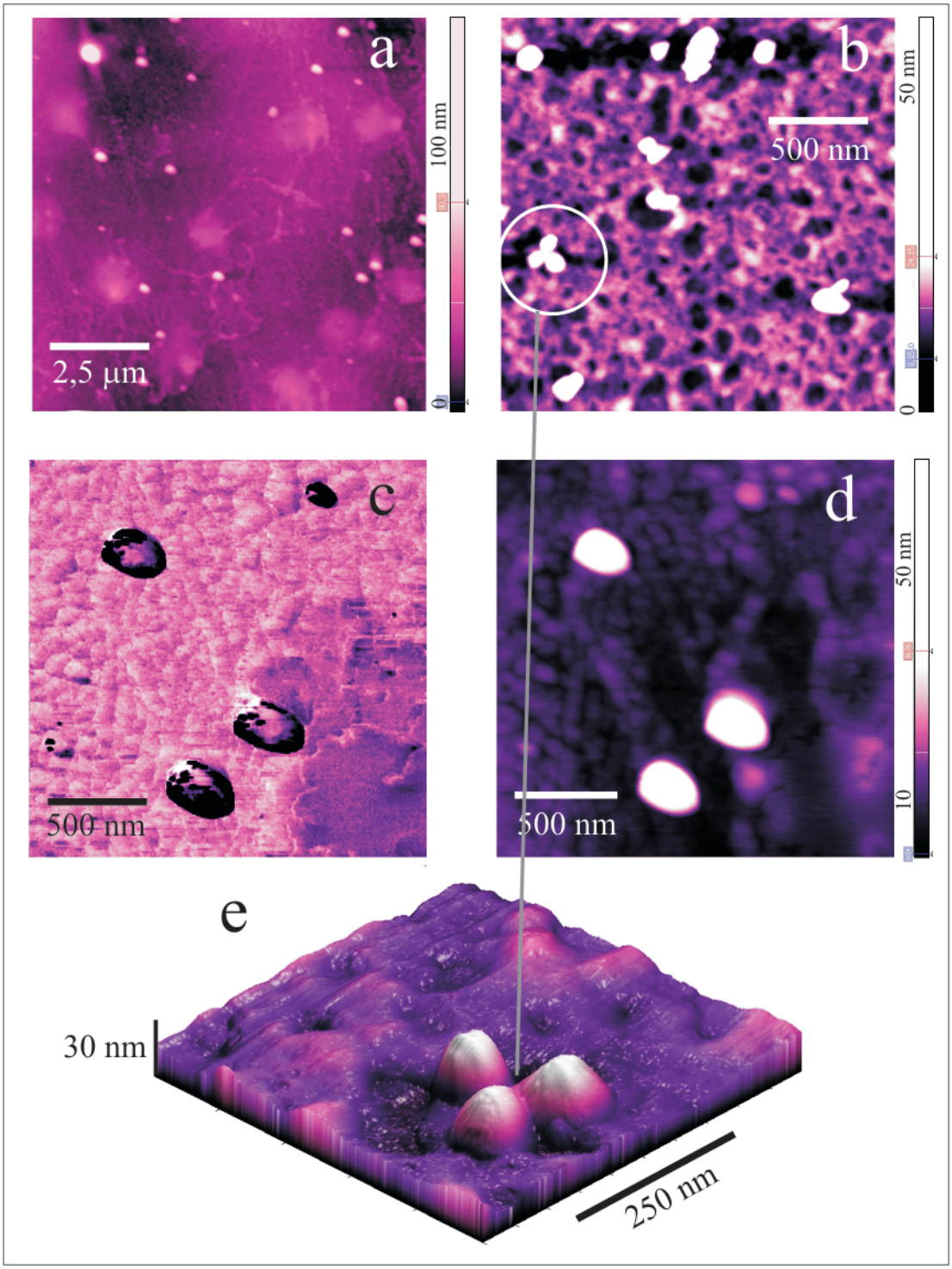

Fig. (1). Atomic force micrograph of the sample of microvesicle-rich blood plasma of healthy donor H1. (**a**, **b**, **d**): the height images, (**c**): the phase image, (**e**): enlarged three dimensional representation of the region marked in (**b**).

Fig. (**2**) shows the atomic force micrograph of a sample of microvesicle-rich blood plasma of a healthy donor H2 and a scanning electron micrograph of isolated microvesicles of the donor H3. The height images (**a**, **c**) and the phase images (**b**, **d**) are presented. Fig. (**2a-d**) shows many closely packed microvesicles of a similar size (the sample exhibits uniform height about 30 nm while the estimated lateral dimensions of the structures which are presumably microvesicles is about 150 nm). Boundaries between microvesicles (dark spots) can be seen in the phase images (Fig. **2b**, **d**). The shape of closely packed microvesicles shown in Fig. (**2**) could be affected by the sample dehydration. The SEM image (**e**) reveals globular and tubular structures exhibiting shapes which are characteristic for vesicles (membrane-enclosed entities with no internal structure) and are therefore interpreted as microvesicles.

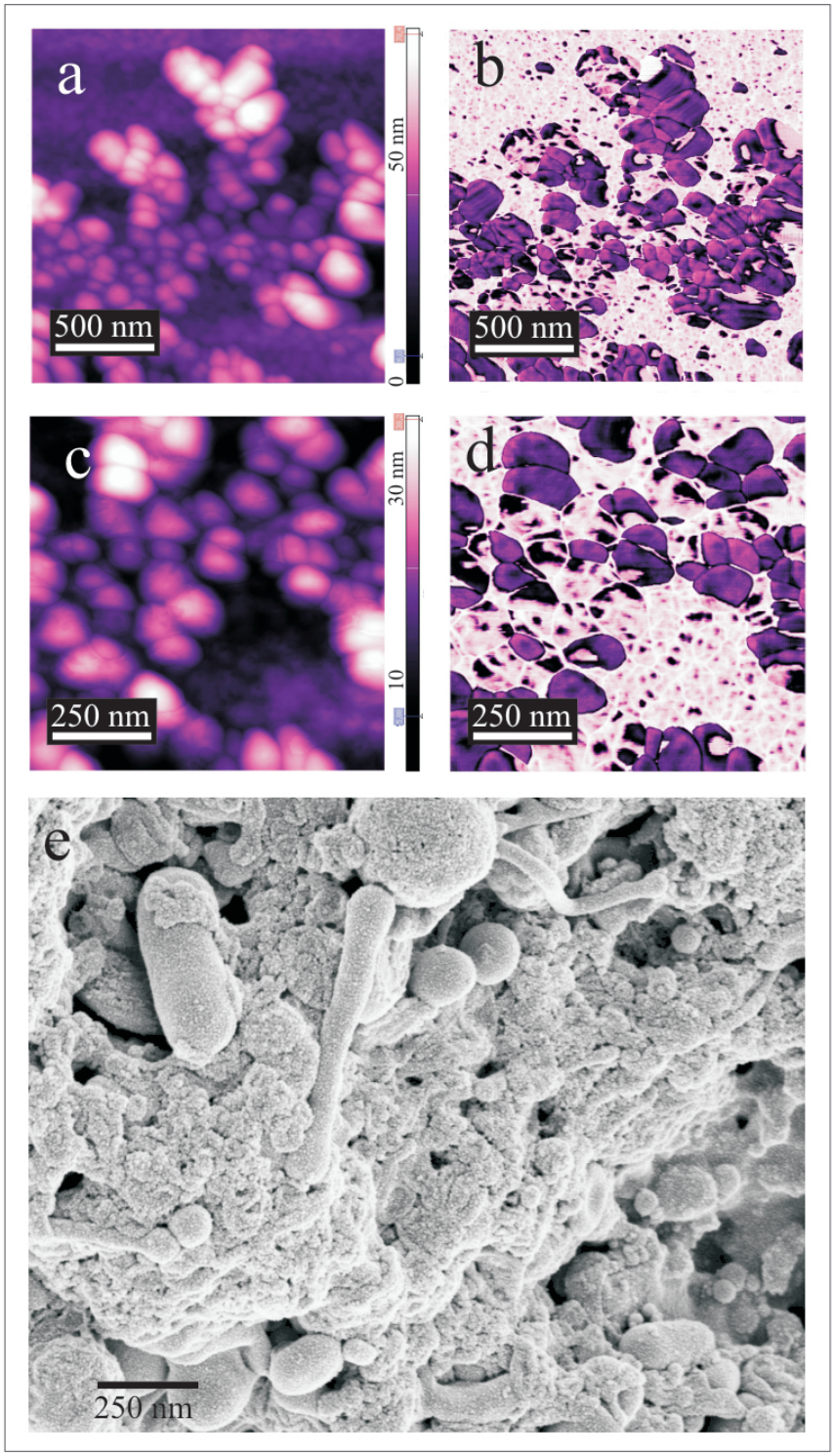

Fig. (2). Atomic force micrograph of the sample of microvesicle-rich blood plasma of healthy donor H2. (**a**, **c**): the height images, (**b**, **d**): the phase images. (**e**): the scanning electron micrograph of a sample of microvesicles isolated from peripheral blood of a healthy donor H3.

Fig. (**3**) shows the flow cytometric scatter diagrams of FS/SC for the blood samples obtained from healthy donors H1 and H2 (Figs. **1** and **2**). Three regions of events were distinguished. The region R1 corresponds to calibration fluorospheres with diameter 1 μm. The events in the region R2 correspond to structures with the same dimensions but different contents of particles yielding different scattering of light. We ascribe these events to the presence of platelets, however, elongated microvesicles may also contribute. Smaller structures ascribed to the region R3 (down to about 50 nanometers) are interpreted as microvesicles and protein-lipid assemblies. Both samples (H1 and H2) show similar distributions of events (Table **1**).

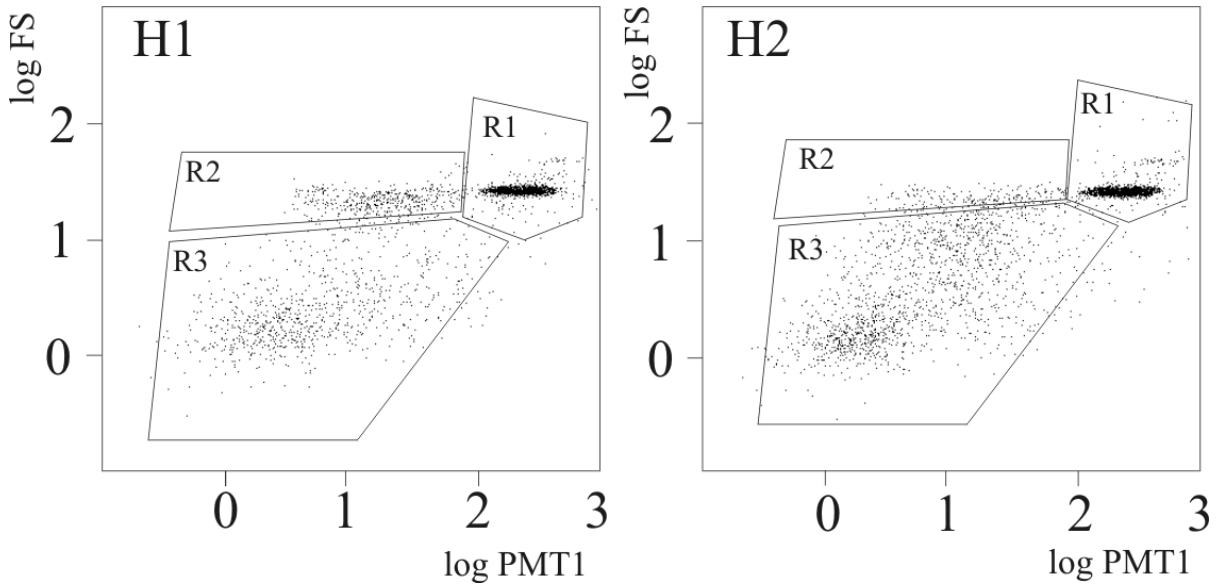

Fig. (3). The flow cytometric scatter diagrams (forward scatter/side scatter) of the material isolated from peripheral blood of healthy donors H1 and H2: calibration fluorospheres with diameter 1 μm (R1) and particles obtained by the isolation procedure (R2-R3).

Table 1. Distribution of Events Recorded by Flow Cytometry of the Isolated Material Obtained from Peripheral Blood of Healthy Donors (H1 and H2). Events in the Region R1 are Interpreted as Calibration Fluorospheres, Events in the Region R2 are Interpreted as Platelets and Elongated Microvesicles, Events in Region R3 are Interpreted as Microvesicles and Protein-Lipid Assemblies

Subject	R1 (%)	R2 (%)	R3 (%)
H1	54	19	26
H2	50	17	31

Fig. (**4**) shows the atomic force micrograph of the sample isolated from synovial fluid of the patient with psoriatic arthritis (P1) and the scanning electron micrograph of the sample isolated form synovial fluid of the patient with rheumatoid arthritis (P2). The height images (**a**, **c**) and the phase images (**b**, **d**) are presented. The concentration of microvesicles isolated from synovial fluid was much higher than the concentration of microvesicles in the samples obtained by isolation from peripheral blood. Therefore the samples obtained from synovial fluid had to be diluted for AFM analysis. From AFM images we observed grain-like structures of different sizes; their height was about 50 nm. The estimated lateral dimension is between 100 and 150 nm. On the other hand, high density of the sample is favorable for SEM. The SEM image (Fig. **4e**) shows many globular structures, which however have more irregular shape compared to microvesicles isolated from blood (Fig. **2e**). Grains observed from AFM images (Fig. **4a-d**) appear smaller in comparison to microvesicles observed by SEM (Fig. **4e**), however the characteristics of the shapes imaged by AFM and SEM seem alike. Furthermore, the grain-like character of shape is revealed in both samples obtained from synovial fluid although they derive from different patients.

DISCUSSION

Due to their small size, membrane constituents can induce strong curvature in the membrane which forms protrusions and eventually microvesicles. Due to this potential for strongly curving the membrane the pinched off microvesicles are very small and can therefore not be observed directly under the optical microscope. Other techniques are required to reveal their shape. Here we present images obtained by atomic force microscope and scanning electron microscope.

AFM and SEM are appropriate techiques to observe biological material [33, 34]. By operating AFM in tapping mode we reduced the friction and adhesive force and enabled imaging of soft biological samples. The height of microvesicles determined from AFM was about 25-60 nm which is considerably smaller than their diameter (about 50-200 nm) (Figs.**1**, **2** and **4**). To some extent this could be attributed to AFM tip broadening [35], but most probably due to the collapse of microvesicles during the drying process. This is supported also by SEM analysis, which yields the diameter of comparable dimensions. However, SEM micrographs reveal that the globular structure of microvesicles was conserved reflecting the preparation process which prevents collapse of the microvesicles.

Imaging of microvesicles by AFM in phase mode gives further information about their properties. In phase mode imaging the phase shift of the oscillating cantilever relative to the driving signal is measured and correlated with specific properties of the material, which depend on the cantilever tip-sample interaction. Therefore the phase shift can be used to differentiate areas by friction, adhesion, and viscoelastic-

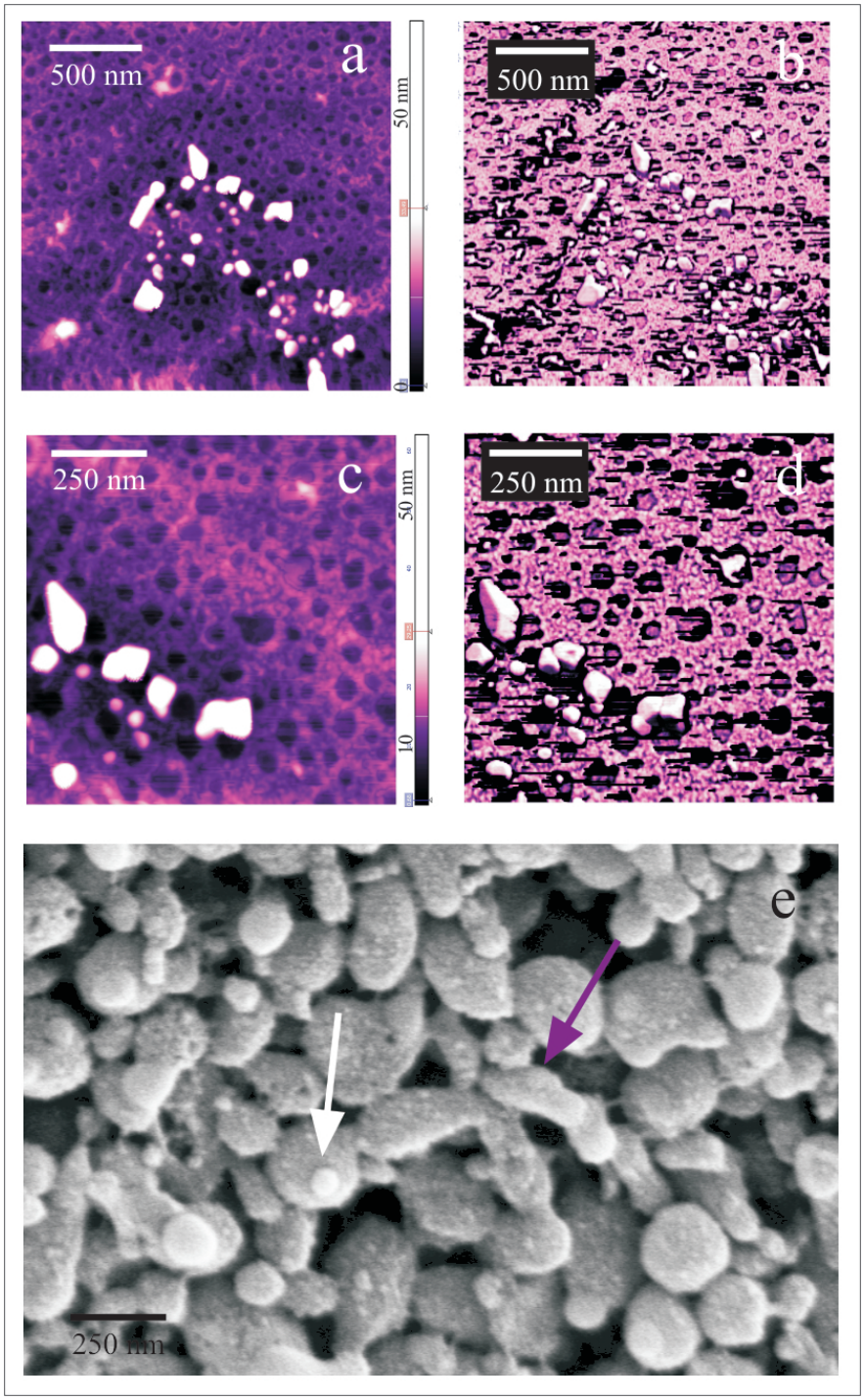

Fig. (4). Atomic force micrograph of the material isolated from synovial fluid obtained from the patient with psoriatic arthritis P1. (**a**, **c**): the height images, (**b**, **d**): the phase images. (**e**): the scanning electron micrograph of the material isolated from synovial fluid of the patient with rheumatoid arthritis P2. Globular structures with high degree of symmetry could be microvesicles (white arrow) while more irregular structures could be also protein-lipid assemblies (violet arrow).

ity. In our study this enabled us to distinguish between individual microvesicles by detecting their boundaries (the dark parts in Figs. **2b** and **d** and Figs. **3b** and **d**).

In analyzing samples (microvesicle-rich plasma, isolated plasma microvesicles/synovial microvesicles) both techniques revealed presence of globular structures which can be according to their size and shape interpreted as microvesicles. To complement the description of the samples, flow cytometry is used, as this method is able to record a very large number of microvesicles and is therefore convenient for obtaining information on the size distribution. Flow cytometry of the same samples shows that the structures observed by AFM and SEM could correspond to the pool of microvesicles.

In analyzing the sample obtained from synovial fluid we observed many small structures which could be microvesicles. However, due to somewhat irregular shapes of these grains, which are revealed by the AFM as well as by SEM we cannot decisively claim that all of these structures are microvesicles. Grains could also be formed by assembly of proteins and lipids. It was determined by flow cytometry [32] that most of the synovial grains derive from leukocytes. Further experiments are needed to reveal the morphology of these grains such as the transmission electron microscopy which could give information on the contents of the grains.

Vesicular structures are expected to attain shapes according to the minimum of the free energy of the membrane. The shapes of vesicular structures were observed in artificial systems composed of phospholipid membranes and also studied theoretically [36], and were found to exhibit a high degree of symmetry. In the light of these experiences one can conclude that globular sphere-like and tube-like structures correspond to microvesicles while the identity of the more irregular structures is still unresolved.

It the protocol for isolation of microvesicles which was used in our work, microvesicles are expected to originate from *in vivo* conditions, however, it is also possible that some of them are formed due to the processing of the sample. Microvesicles could be formed due to the collection of the sample, due to the presence of the anticoagulant in the tubes, due to vesiculation of cells in the time (minutes) between the collection and centrifugation, due to pipetting and during centrifugation. It is yet unclear to what extent these causes contribute to the pool of microvesicles in the sample. The observed samples should therefore be considered as subject to the *in vivo* conditions as well as of the isolation protocol. It was one of our aims to visualize the sample obtained with the particular protocol [22] used in clinical studies to provide additional information to the data obtained by flow cytometry. Nevertheless, our experience indicates that following the same processing procedure the number of microparticles measured by flow cytometry significantly differs between individuals as well as between groups of patients [37] which indicates that an important (probably the major) pool of microvesicles derives from *in vivo* conditions.

Microvesicles were found to stimulate cells by transfering surface-bound ligands and receptors between cells [12, 38-40] and deliver biologically important contents such as molecules [12, 41] and infectious particles [42] into cells [16]. Therefore, microvesicles were suggested to play an important role also in mechanisms underlaying autoimmune diseases. It was shown that melanoma as well as ovarian cancer cell - derived microvesicles contain in their membrane Fas ligand and can upon interaction with Fas receptor - carrying T cells induce apoptosis of T cells [11, 43]. Therefrom, a possible mechanism of tumor progression was suggested by allowing tumor cells to escape immunosurveillance [44]. Based on the evidence on the microvesicle-mediated transport of material between cells, a possible mechanism of platelet destruction in immune thrombocytopenia was suggested. It was hypothesized that microvesicles shed from platelets of patients with immune thrombocytopenia may transfer platelet derived-antigens involved in the formation of anti-platelet antibodies to other cells and thus "expand" the population of target cells that may be affected by anti-platelet antibodies [16]. Increased levels of circulating microparticles were found in patients with acute heparin-induced thrombocytopenia compared with controls [44] and in patients with systemic lupus erythematosus [44], which was connected to thrombotic complications observed in some patients with these disorders [44, 45]. In most reported disorders including autoimmune diseases, microvesiculation was found to be enhanced. It seems that there is a feed-forward mechanism of microvesicles as indicators as well as promotors of the disorder.

Interplay between microvesiculation and composition of the surrounding solution is of special interest since it may reveal the role of different molecules such as antibodies in the process. Vesiculation was studied in artificial membranes, i.e. giant phospholipid vesicles. These structures are large enough to be observed directly under the phase contrast microscope. Budding of the membrane and pinching off the bud can be induced in a controlled way by changing the temperature or adding substances to the suspension containing vesicles. It was observed that a cofactor in antiphospholipid antibody binding to phospholipids - β2-gylcoprotein I - can mediate attractive interaction between negatively charged and neutral membranes [46-48] in the dose-dependent manner [49]. If the effect is strong enough, the bud adheres to the mother membrane and does not become free microvesicle [47]. With this respect, β2-glycoprotein I would be anticoagulant which is supported by clinical experience [50]. Also it was found that antiphospholipid antibodies may suppress the mediating effect of β2 glycoprotein I [46], thereby exhibiting their role as procoagulants.

In pursue for understanding of autoimmune mechanisms, many works have been devoted to biochemical aspects. However, biophysical methods that have recently been given attention point to nonspecific interactions and their origins [51-53]. We believe that approaches with different points of view will help to improve understanding of microvesiculation and related phenomena.

To conclude, we have observed the material isolated by one of protocols which is used for clinical studies, by AFM and SEM. Both, material isolated from peripheral blood and material isolated from synovial fluid contain structures in the range between 50 and 300 nanometers with characteristic vesicular shapes. However, other shapes are also present, especially in the samples of synovial fluid. Further studies and complementary techniques are required for decisive interpretation of the identity of particles in the isolated material.

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