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Synovial fluid protein adsorption on polymer-based artificial hip joint material investigated by MALDI-TOF mass spectrometry imaging

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ABSTRACT

UHMW-PE (ultra-high molecular weight polyethylene), most frequently used material in acetabular cup replacement, is affected by the interaction with its surrounding synovial fluid. It is assumed that protein layer formation is of high importance for lubrication, however alters polymer characteristics. This study investigates *in vitro* protein adsorption on gamma-irradiated and Vitamin E doped UHMW-PE using synovia as modeling system. SDS-PAGE and MALDI-TOF mass spectrometry imaging showed adsorption of high abundance proteins in a mass range between 2 and 200 kDa. Protein layer formation was observed on planar UHMW-PE material, whereas morphologically modified UHMW-PE regions were highly affected by protein aggregation.

1. Introduction

Ultra high molecular weight polyethylene (PE-UHMW) is a very frequently used material in artificial joint replacement systems. It provides easy usability, extreme high mechanical wear resistance, high biocompatibility and is to a certain extent self-lubricating [1]. However, the number of revision surgeries is still high, sometimes necessary because of the rapid shelf life aging process and *in vivo* degradation due to mechanical wear, oxidation and material modification [2–4]. Synovial fluid (SF), the major lubricating system in the joint compartment, directly interacts with the material surface, leading to biomolecule adsorption and diffusion [1,5] possibly altering the polymer characteristics and stability. The major components have already been investigated, however it has not been determined yet, whether modified PE-UHMW surface areas lead to enhanced biomolecule adsorption and diffusion or *vice versa*. Furthermore, protein layer formation on PE-UHMW has so far not been investigated except for a

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few selected protein and carbohydrate species, *e.g.* albumin and hyaluronic acid [6]. It is known that the major abundant components of synovial fluid, *e.g.* hyaluronic acid, albumin or immunoglobulin, interact with PE-UHMW and tend to adsorb on the surface in model fluid systems. However, the interaction of the whole synovial proteome in the presence of lipids *etc.* with PE-UHMW has never been investigated. Because the synovial fluid composition is very similar to blood plasma (except for the high hyaluronic acid content) it is also highly affected by the patient's age, sex, life style and pathological status [7,8]. Therefore it is of high interest, which proteins except albumin and immunoglobulin in fact adsorb on PE-UHMW before a deeper understanding of the pathological process can be obtained and/or material related selective adsorption effects can be gathered.

Different compositions of PE-UHMW, Vitamin E doped material, highly cross-linked PE-UHMW and materials undergoing different kinds of sterilization strategies have already been tested in clinical studies for improving the materials' properties and reducing protein adsorption. Highly abundant glycoproteins have been shown to adsorb on PE-UHMW in simulating models and adsorption has been investigated to occur unspecific and concentration independent [1]. In the same study model fluids containing only one glycoprotein of interest revealed that the formed protein layer enhances the lubrication and friction behavior of PE-UHMW. However, besides the selected glycoproteins also other high abundance and acute phase proteins occurring during inflammation and oxidative stress, *e.g.* during rheumatic diseases, are relevant for adsorption, friction behavior and consequently material modification.

The presented study focuses on the unbiased identification and localization of proteins present in synovial fluid adsorbing on different varieties of PE-UHMW material. To answer both questions, protein identity and spatial distribution, mass spectrometry imaging (MSI) by means of matrix assisted laser desorption/ionization time-of-flight (MALDI-TOF) examination was correlated to SDS PAGE analysis of adsorbed proteins. MSI is a well-established method for the localization and identification of analytes of interest within an untargeted approach [9]. To date MSI has been applied to a huge variety of biological samples [10–12] and it has to be mentioned that surface analysis of biomaterials is an exponentially growing field gaining more importance for the analysis of lubrication interaction and material modifications [13,14].

One limiting fact of MSI is that protein identification in the high molecular mass range is only possible based on the tentative assignment of proteins to measured *m/z* values, respectively the molecular weight of detected molecules [15]. To corroborate protein assignment after MSI analysis, SDS PAGE analysis was chosen, providing the possibility to compare synovial protein patterns to patterns of protein compounds adsorbed on PE-UHMW of different compositions with respect to overall detected proteins. In parallel MS-based protein identification after *in-gel* digestion is feasible. Protein identification for adsorbed molecules was furthermore verified by *on-tissue* ("on polymer") digestion [16,17]. The hydrophobic surface of PE-UHMW supports the preservation of protein localization during trypsin application in aqueous buffer systems, however, diffusion is a critical point for both enzyme and matrix application. To obtain reliable results homogeneous layers of trypsin and MALDI matrix solution were applied using a piezo printer (Chemical Inkjet Printer, ChIP-1000 [18]) to the protein-carrying PE-UHMW material.

2. Experimental

2.1. Materials

The Institute of Materials Science and Technology, Vienna University of Technology, provided gamma-irradiated PE-UHMW (GUR-1050 PE-UHMW) and PE-UHMW samples doped with Vitamin E. All chemicals and reagents, unless indicated specifically, were from Sigma–Aldrich (USA) with a purity of at least 99% if not stated otherwise. Ultrapure water (uH_2O) was obtained from a Simplicity system (Millipore, USA) with a specific conductivity of $\Omega_m \leq 18$ S/cm. Human synovial fluid (SF), sampled after joint revision, was provided by the Institute of Materials Science and Technology.

2.2. Sample preparation

Virgin gamma irradiated GUR-1050 PE-UHMW and Vitamin E doped PE-UHMW samples were used. PE-UHMW was cut into small blocks of comparable geometry ($m = 85 \pm 2$ mg, $V \approx 125$ mm^3), which were either used for incubation experiments or sliced to 15 μm using cryostat (CM1950, Leica, Germany) and mounted on indium-tin oxide (ITO) coated glass slides (Sigma–Aldrich) using conductive tape (Shimadzu, Japan). The final polymer slice can exhibit folded regions after attachment. However, those regions were used to simulate uneven and rough regions and edges, as they actually occur *in vivo*, caused by mechanical wear and abrasion processes in the acetabular cup. All PE-UHMW samples were incubated in SF at 37 °C for 24 h. After incubation, samples were rinsed with uH_2O and vacuum dried.

For MS and MSI experiments standard MALDI matrices, α-cyano-4-hydroxycinnamic acid (HCCA) and sinapinic acid (SA) were dissolved in 70% acetonitrile (ACN), 30% uH_2O (v/v) containing 0.1% trifluoroacetic acid (TFA) at a concentration of 13 (HCCA) and 25 (SA) mg/mL. Automatic matrix deposition was performed using a chemical inkjet printer ChIP-1000 (Shimadzu) or a commonly used airbrush device (Conrad, Germany). For the airbrush application of MALDI matrix a working distance of 10–12 cm was chosen with an approximate angle of 50–60° covering the sample in several iterative steps.

2.3. MS and MSI analysis

MSI experiments were performed on a MALDI-TOF/RTOF instrument (UltrafleXtreme, Bruker Daltonics, Germany) in positive linear or reflectron mode, equipped with a 355 nm SmartBeam laser pulsed at 2000 Hz [19] and on a MALDI-TOF/RTOF instrument (AXIMA TOF^2, Shimadzu Kratos Analytical, UK) equipped with a 20 Hz nitrogen (337 nm) laser. At a spatial resolution of 100–150 μm, mass spectra based on 1000 (AXIMA TOF^2) or 2000 (UltrafleXtreme) single laser shots were acquired per position over an average sample size of

8000 single imaging positions for polymer samples (approx. 8 × 8 mm) if not otherwise stated. For image reconstruction FlexImaging v. 3.0 software (Bruker Daltonics) and Biomap 38.04 (Novartis, Switzerland) was used.

2.4. Gel electrophoresis

For protein extraction 200 μL SF, PE-UHMW blocks after incubation in synovia and blank material was deposited in 1200 μL uH_2O before 100 μL trichloroacetic acid (TCA; 20%) were added 5 times and vortexed thoroughly between each single addition. Samples were incubated for 30 min at 4 °C and centrifuged at 14,000 rpm and 4 °C for another 30 min. The supernatant and polymer blocks were removed and the protein pellet re-dissolved in 10 μL lysis buffer (50 mM Tris/HCl pH 8.0, 2 mM dithiothreitol, 10% v/v glycerol, 0.5% v/v Tween 20, 10% toluol). For protein precipitation with ethanol, 50 μL SF or the PE-UHMW blocks were mixed with 450 μL precooled ethanol (4 °C) and incubated at −70 °C for 2 h before centrifugation at 14,000 rpm and room temperature for 30 min. The polymer blocks and the supernatant were removed and ethanol residues were evaporated in the vacuum centrifuge. After drying the pellet was re-dissolved in lysis buffer. Protein concentration was determined according to Bradford [20].

For SDS PAGE analysis protein pellets were dissolved in lithium dodecyl sulfate sample buffer (26.5 mM Tris–HCl, 35.25 mM Tris Base, 0.5% lithium dodecyl sulfate, 2.5% glycerol, 0.1275 mM EDTA, 0.055 mM SERVA Blue G250, 0.044 mM Phenol Red, pH 8.5, 50 mM dithiothreitol) at 95 °C for 5 min and applied to a precast NuPAGE 4–12% Bis-Tris polyacrylamide gel (Invitrogen, USA). Electrophoresis was conducted at 125 V (60 mA max., 12.5 W) in a XcellSurelock Mini Cell electrophoresis system (Invitrogen, USA). Gels were silver stained [21] for protein detection.

2.5. Protein identification

Proteins were either identified after SDS PAGE by *in-gel* or by *on-tissue* ("on polymer") digestion. For the first, gel bands were excised with a clean scalpel and destained using 100 mM $Na_2S_2O_3$ and 30 mM $K_4Fe(CN)_6{\cdot}3H_2O$ (1:1, v/v). Gel pieces were treated with ACN and rehydrated with 100 mM NH_4HCO_3. After reduction (10 mM DTT in 100 mM NH_4HCO_3) and alkylation (50 mM iodoacetamide in 100 mM NH_4HCO_3) the gel pieces were dried in a vacuum centrifuge and rehydrated in approx. 10 μL 50 mM NH_4HCO_3 (pH 8.5) containing 5% ACN and 125 ng trypsin (porcine, proteomics grade, Roche, Switzerland). Digestion was carried out for 24 h at 37 °C. Peptides were extracted with 50 mM NH_4HCO_3/ACN (1/1, v/v) and two times with uH_2O/ACN containing 0.1% TFA (1/1, v/v), for 15 min each. All extracts of one respective spot were pooled and dried in a vacuum centrifuge. After reconstitution in 0.1% TFA peptides were desalted using C_{18} ZipTips (Millipore, USA) and eluted with 5 mg/mL HCCA prepared in ACN/0.1%TFA (50/50, v/v) in the final step. Peptide mass fingerprinting (PMF) and sequence tag analysis were carried out on a MALDI-TOF/RTOF instrument (UltrafleXtreme).

For "on polymer" enzymatic treatment, the ChIP-1000 was used for precise trypsin deposition at a standard concentration of 3 ng/μm^2 containing 1% Rapigest (Waters, USA) dissolved in 50 mM NH_4HCO_3 (pH 7.5) containing 5% ACN. Trypsin solution was applied at a lateral resolution of 100 μm covering the complete sample area. For digestion samples were stored at 37 °C for 24 h in saturated atmosphere (ethanol/water, 50/50, v/v, 80% relative humidity). After incubation samples were carefully washed with uH_2O to remove salts, delocalized peptides not adsorbed to the polymer and autolytic fragments from trypsin. Samples were vacuum dried for 15 min and HCCA was applied afterwards as described.

For all enzymatic digestion data, autolytic tryptic products, keratin and blank artifacts were assigned and removed before database search (SWISSPROT, December 2013) using Mascot [22] with the following parameters: taxonomy *Homo sapiens*, monoisotopic mass values, peptide mass tolerance of ±0.3 Da (for PMF and PSD experiments), 2 missed cleavages, a fixed modification carboxyamidomethylation and methionine oxidations set as variable modification.

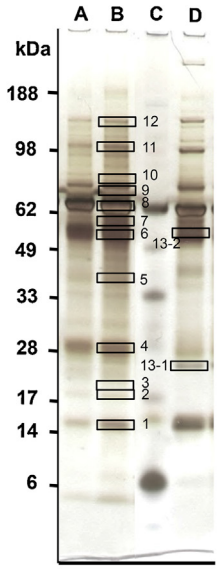
3. Results

3.1. Protein identification on PE-UHMW

Two different PE-UHMW materials were investigated concerning protein adsorption: conventionally used GUR-1050 PE-UHMW and Vitamin E doped PE-UHMW, which is claimed to be more resistant to oxidative degradation [5]. After incubating the two different materials in the same synovia at 37 °C for 24 h, adsorbed proteins were extracted. Similar to plasma, 85% of synovia consists of the same well-described high abundance proteins. However, to identify proteins actually adsorbing in a concerted manner from synovia on PE-UHMW in the bio-compartment, we decided against special synovia treatment (*e.g.* depletion) well aware of the possibility to identify exactly those high abundance proteins.

For this approach, two different extraction methods were evaluated: an ethanol and a TCA based extraction methods (details see Section 2.1). Protein concentrations were determined to compare the efficiency of both approaches. The protein concentration obtained according to Bradford differed significantly yielding 14.68 ± 4.24 μg/μL for the ethanol (n = 15) based method and 7.74 ± 1.89 μg/μL for the same polymer type extracted with TCA (n = 15). However, no qualitative differences were observed for the SDS PAGE protein pattern and the number of protein lanes was comparable in the apparent molecular mass range between 3 and 200 kDa. For proceeding experiments TCA precipitation was applied, in order to avoid eventual lipid or glycan contamination (easily extracted with ethanol) and based on the higher reproducibility.

Protein analysis from the two different PE-UHMW species revealed an average protein concentration of 9.46 μg/μL for GUR-1050 PE-UHMW after extraction with TCA and 13.08 μg/μL for Vitamin E doped samples. Applying concentration-matched samples to SDS PAGE revealed lighter stained protein bands for the Vitamin E doped samples. More than 10 high abundance proteins could be identified after *in-gel* digestion (Fig. 1).



Protein ID (Gel band number)	molecular weight				
	Literature [kDa]	SDS-PAGE [kDa]	MALDI profiling [m/z]	Peptides associated	Carbohydrate [%]
Fibrinogen	340	n.d.	n.d.	n.d.	3
IgG (13-1, 13-2)	150	28(13-1) / 50(13-2)	147421	8	3
Ceruloplasmin (12)	135	140	133255	25	7
Haptoglobin (11)	104	103	100399	4	16
Plasminogen	81 / 90.1 (MADLI)	n.d.	88940	20	3
Transferrin (10)	79.1	77	78052	23	6
Comp C3-b (9)	75	75	73956	9	**
Albumin (8)	66.4	68	66615	18	x
Vitronectin	65-75 / 59.8 (MALDI)	n.d.	58595	9	30
α1-Antichymotrypsin (7)	52.3	55	53942	9	27
Apo A-4 (6)	43.4	n.d.	44501	21	x
α1 Acid glycoprotein	40 / 33-36 (MALDI)	40	n.d.	8	45
Apo E (5)	34.2	35	33302	18	x
Apo A-I (4)	28.1	29	29528	19	x
Apo D (3)	22.5	23	22301	2	x
Apo H-III (HDL) (2)	17	18	16656	n.d.	x
Transthyretin (1)	13.8	15	13659	1	x
Apo C-II	8.9	n.d.	8894	1	x
Apo C-III	8.8	n.d.	8740	n.d.	0-15
Apo A-II	8.7	n.d.	8592	3	x
Apo C-I	6.6	n.d.	6343	1	x*

n.d.: not detected, x* no glycosylation, **no available information

Fig. 1 – Summary of SDS PAGE analysis and protein identification of high abundance proteins in synovia and proteins recovered from PE-UHMW surfaces after incubation in synovia for 24 h. SDS PAGE: proteins recovered from Vitamin E doped PE-UHMW (lane A), GUR-1050 PE-UHMW (lane B), Seeblue Prestained Marker (lane C) and synovial fluid extracts (lane D). Table: proteins identified after *in-gel* digestion in comparison to *m/z* values detected on PE-UHMW (GUR-1050 and Vitamin E doped) by MALDI profiling. Number of peptides derived by "*on-polymer*" digestion associated to respective proteins and degree of glycosylation listed in the database.

Compared to protein extracts from SF, it can be seen that all high abundance proteins adsorb on both PE-UHMW types. Relative protein intensities within the protein extracts are comparable except for the light chain of IgG, which seems to be less abundant on the polymers compared to plain synovia.

Aside from gel electrophoretic analysis, adsorbed proteins were also detected as intact molecules on the polymer surfaces using MALDI-TOF MS (MALDI profiling) after washing the polymer with uH_2O. Synovia contains a very high amount of salts, therefore the washing procedure had to be repeated if salt residues were still visible as small crystals under the light microscope. HCCA and SA were deposited directly on the washed polymer using the airbrush instrument. At this point the maintenance of localization of the proteins was not of importance. Thin MALDI matrix layers were obtained on the very hydrophobic surface using both matrices with ACN/0.1% TFA at a ratio of 70:30 (v/v) as solvent. For MS profiling experiments the whole polymer area, approx. 25 mm^2, was measured with a laser spot diameter of 50 μm and a broad energy profile to avoid polymer degradation (all experiments performed on the UltrafleXtreme). Fig. 2 shows the protein pattern from GUR-1050 PE-UHMW surface for two different mass ranges accumulating 5000 shots for each spectrum. HCCA proved successful for the molecular mass range between 2 and 20 kDa and as expected SA performed best for the higher range, 20–200 kDa. In both cases $[M]^+$ ions were generated. Even though PE-UHMW is a highly insulating material, interfering therefore with the MALDI process itself, ion intensities and mass resolution were sufficient for unambiguous peak assignment. The table in Fig. 1 illustrates that after incubation with SF the determined *m/z* values most likely correspond to apolipoproteins of different classes (Apo C-I, Apo A-II, Apo C-III, Apo C-II, Apo D, Apo A-I, Apo E, Apo A-4), transthyretin, α1-antichymotrypsin, albumin, complement C3-b, transferrin, haptoglobin, ceruloplasmin and the light as well as heavy chain of IgG. They were all detected on both, GUR-1050 PE-UHMW and Vitamin E doped PE-UHMW material.

SDS PAGE and MALDI profiling showed that all identified proteins and detected signals are belonging to high abundance proteins present in SF. However, it is observed that many of the tentatively assigned apolipoprotein subunits are present in MALDI profiling analysis alone. This can either be explained by the fact that this lower molecular weight protein fraction is not accessed in conventional SDS PAGE, or that these subunits are specifically attaching to the polymer surfaces and cannot be removed by protein precipitation using TCA. It has to be mentioned that only a small percentage of the proteins identified after *in-gel* digestion represent glycoproteins (see table in Fig. 1) and that from this no clear conclusion can be drawn whether glycoproteins or otherwise post-translationally modified proteins are preferentially adsorbed to the PE-UHMW material. The same conclusion can be drawn for the feature protein hydrophobicity. It was also observed that SDS PAGE band intensities gave no unambiguously hint to the assumption that the size of a protein has a significant effect on adsorption.

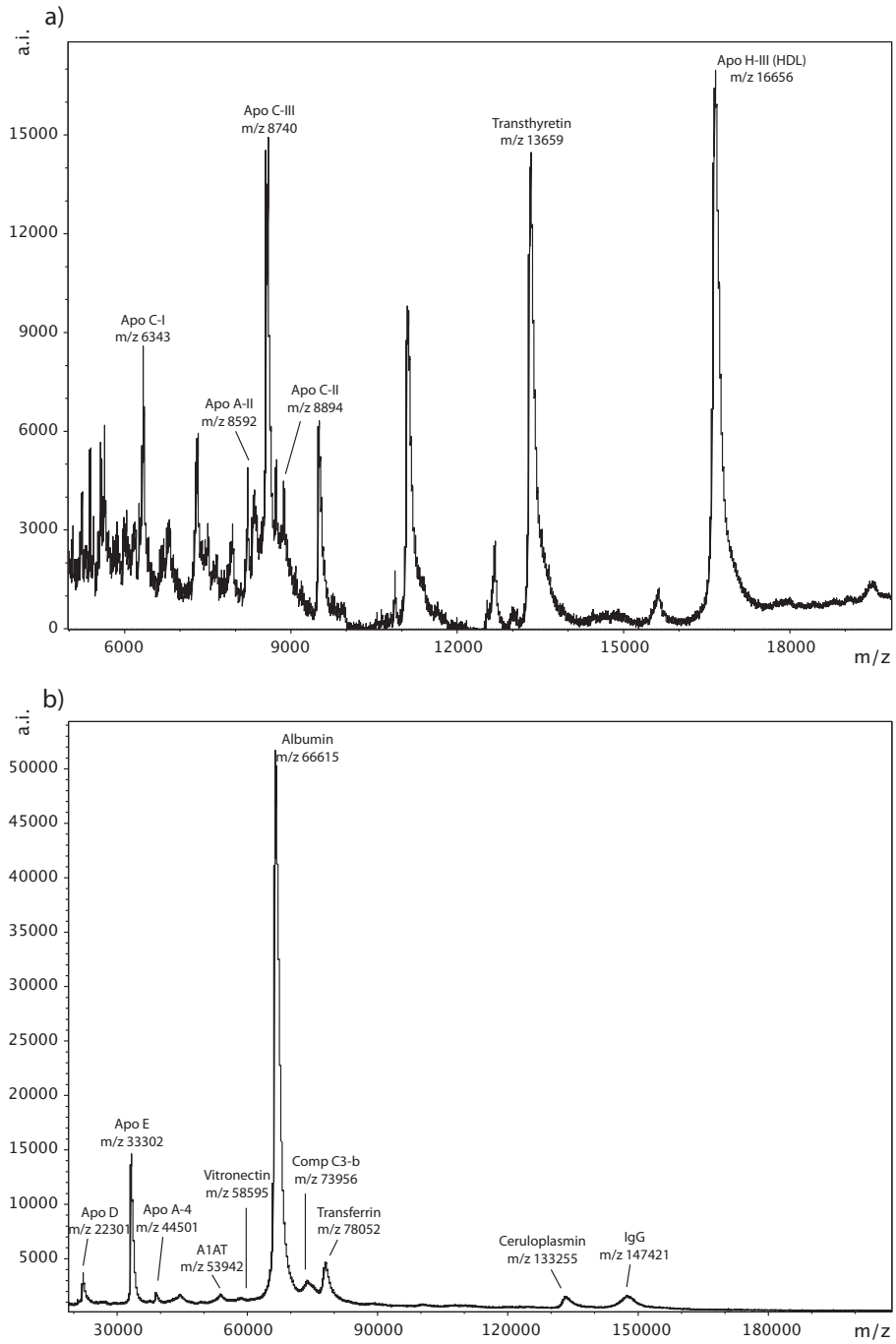


Fig. 2 – Positive ion MALDI mass spectra obtained by MS profiling of GUR-1050 PE-UHMW incubated in synovia for 24 h at 37 °C. (a) HCCA was used as matrix for the molecular mass range between 2 and 20 kDa and (b) SA was used as matrix for the molecular mass range between 20 and 200 kDa.

3.2. *MALDI matrix application on hydrophobic surfaces for MSI experiments*

Investigating protein adsorption on polymer surfaces by MALDI-TOF MS requires thorough optimization of sample preparation particularly due the low amounts present. The most critical step is the generation of a homogenous MALDI matrix layer on a large sample surface. The hydrophobic surface of the PE-UHMW samples favored the formation of a liquid film if the matrix was not carefully applied droplet by droplet. Especially folded regions, which can occur after applying the thin polymer samples to the ITO slides after cryomicrotomy, provide problems regarding matrix crystallization. MALDI matrix solution application with slowly evaporating solvents leads to a matrix flow leaving no matrix on folded areas but large crystals on planar regions. This can ofcourse favor disadvantageous analyte diffusion. Additionally it is known that protein desorption/ionization during the MALDI

process is more effective after incorporating analytes in solvent systems containing acidic components, as a consequence of efficient protonation. On the polymer surfaces, however, organic solvent systems were shown in preliminary results to have better crystallization properties, most likely due to their much higher volatility [23]. Further studies showed now that organic solvent systems revealed very small crystal sizes and homogenous covering of the polymeric material, while aqueous solvent systems resulted in improved signal quality but less favorable sweet spot formation in combination with sometimes very small areas covered with high concentrations of matrix. The matrix/solvent system turned out to be performing best with respect to signal quality (signal-to-noise, intensity and mass resolution), surface coverage and applicability was a solution of ACN/aqueous 0.1% TFA at a ratio of 70/30 for both HCCA and SA.

For matrix application the piezo printer (Chip 1000) and the airbrush instrument were compared with special respect to analyte diffusion. To prevent the formation of a thin solvent layer on top of the polymer due to droplets trickling away, the distance between single matrix droplets (approx. 80 pL) applied with the piezo printer was set to 100 μm. To cover the total area of 25 mm^2 long printing operation times were necessary (up to 5 h). For this, analyte diffusion was investigated when using an airbrush system instead of the piezo printer for MALDI matrix deposition to reduce overall operating times. A peptide standard, 500 fM leucine-enkephalin, was deposited on an ITO target with the piezo printer using the area print mode of the instrument, which leads to a complete covering of a pre-defined area. This area was then covered with HCCA as MALDI matrix using the airbrush. We observed a recognizable liquid layer of solvent on the ITO target, which evaporated more slowly than the MALDI matrix/solvent systems applied by the piezo printer. Thus after drying, light microscopy revealed a thin matrix layer with homogeneous crystal distribution. Moreover MSI experiments demonstrated (Fig. 3) that the airbrush represents a matrix application device sufficient for matrix application without significant analyte dislocation at a lateral resolution of 100 μm. The very sharp edged area visualized for the peptide ion emphasizes the accuracy of peptide application by the piezo printer. The applied peptide was perfectly preserved and detected after MALDI matrix application.

3.3. *Visualizing intact protein distribution on PE-UHMW up to 204 kDa*

Besides the finding that proteins adsorb on PE-UHMW aside from their post-translational modifications or hydrophobic quality, it is necessary to localize proteins adsorbing on PE-UHMW in order to correlate them to polymer qualities such as surface roughness or oxidation.

For this the 15 μm PE-UHMW slices, incubated in SF for 24 h, were investigated concerning the adsorbed protein layer and the localization of certain proteins in a MSI approach. HCCA for the low or SA for the high molecular mass range were applied using the piezo printer at a lateral resolution of 100 μm covering the whole PE-UHMW sample homogeneously. Fig. 4a shows an exemplary covered PE-UHMW sample after applying HCCA with the piezo printer. A rather thick matrix layer with fine crystal formation is observed. Similar results were obtained for SA. The same proteins as in profiling experiments were detected, *i.e.* all highly abundant species such as albumin, IgG and the apolipoprotein subclasses.

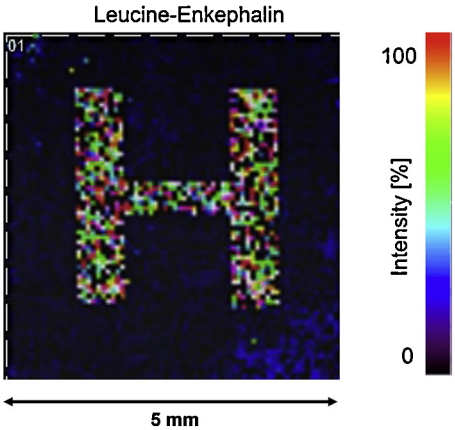


Fig. 3 – Non-normalized intensity distribution of Leucine–Enkephalin (peptide solution on glass slide is not observable, therefore no image provided/transparent) printed with the piezo printer in an "H"-shaped area on transparent ITO glass before HCCA (ACN/aqueous TFA (50/0.1, v/v)) application with an airbrush. Visualization: FlexImaging. Lateral resolution of the MSI experiment: 100 μm.

Profile mass spectra were already presented in Fig. 2a and 2b showing protein adsorption up to 204 kDa. Yet some proteins exhibited characteristic localization on the polymer material. Fig. 4b shows the intensity distribution of apolipoprotein D $[M+H]^+$ (*m/z* 22,301) and Fig. 4e the distribution of the unknown protein at 204 kDa on GUR-1050 PE-UHMW after total ion current (TIC) normalization. Molecules were almost homogeneously distributed on the polymer. The analyzed PE-UHMW sample showed a small folded area in the center, whereas the majority area was mounted completely planar to the conductive tape. On perfectly prepared samples all proteins were detected with a similar homogeneous lateral distributions. Interestingly that in the few areas not covered with Apo D especially albumin was found (Fig. 4c). On both polymer variants, GUR-1050 and Vitamin E doped, *m/z* values correlating to $[M+H]^+$ ions of human serum albumin (*m/z* 66,615) were detected in the center region of the polymer slice, with especially high intensities in folded area (Fig. 4c and f). The area of high intensity on top of the sample results from protein adsorption on the conductive tape on which the polymer is mounted. Compared to the conventionally used GUR-1050 PE-UHMW, Vitamin E doped material (Fig. 4f) showed less signal intensities for all detected proteins. However, adsorbed proteins, IgG and an unidentified compound with *m/z* 204,000 (Fig. 4d and e), revealed similar preferences for folded regions and sharp edges. To investigate the influence of protein degradation due to protease activity, MSI experiments were performed on samples removed from SF incubation after 15, 30, 60 min and 3 h. It was found that homogeneous protein layers are formed already after 30 min of incubation and no loss of signal intensity or

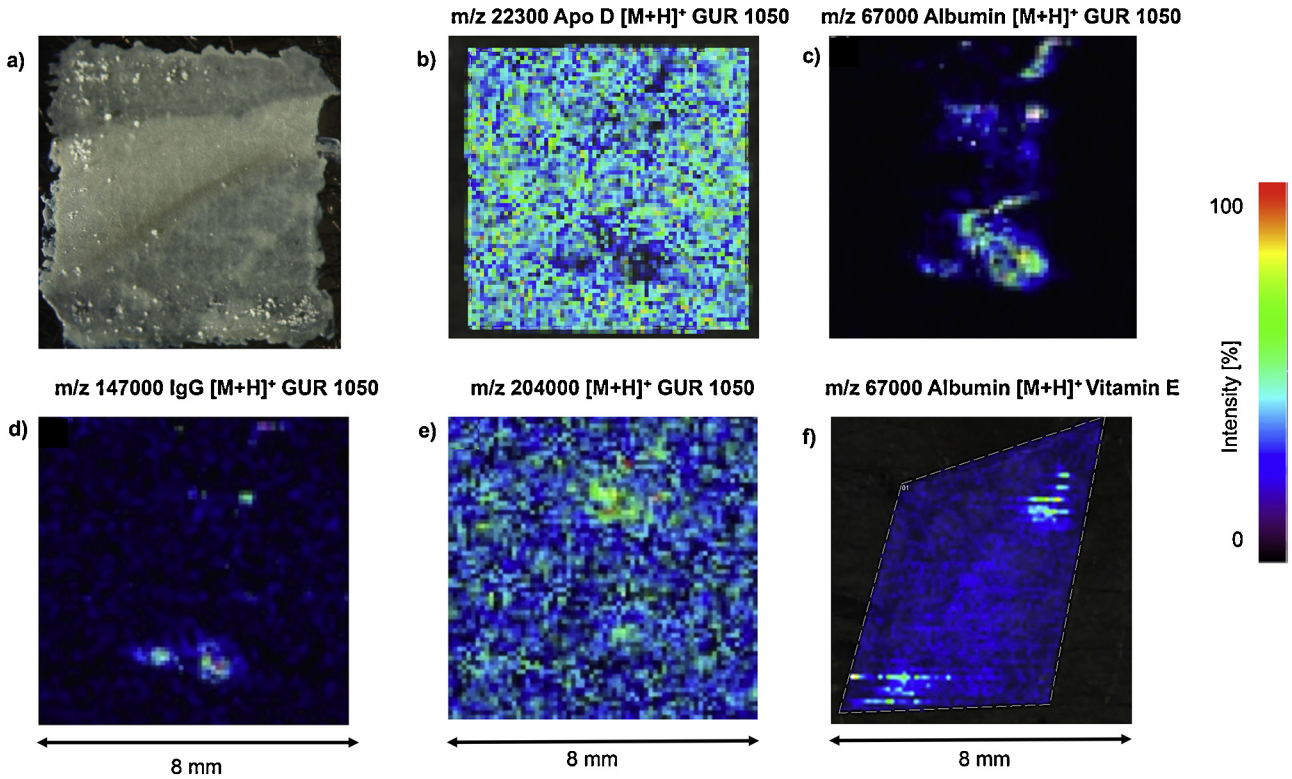


Fig. 4 – MSI experiments of GUR-1050 and Vitamin E doped PE-UHMW incubated in synovia for 24 h at 37 °C. (a) Matrix crystallization on PE-UHMW using HCCA in ACN/0.1% TFA (70/30, v/v). MSI experiments to localize (b) Apo D ([M+H]$^+$, *m/z* 22,301), (c) albumin ([M+H]$^+$, *m/z* 66,615) (d) IgG ([M+H]$^+$, *m/z* 147,000) and (e) *m/z* 204,000 ([M+H]$^+$) on GUR-1050 PE-UHMW (transparent) and (f) albumin [M+H]$^+$ on Vitamin E doped PE-UHMW (transparent) were performed at a lateral resolution of 100 μm.

increased signal-to-noise ratio excelling biological variation was observed (data not shown).

3.4. *Identification of high molecular weight proteins by "on polymer" digestion*

To verify tentative protein identifications on PE-UHMW material, adsorbed proteins have to be enzymatically digested and the generated peptides have to be sequenced by mass spectrometry. To perform enzymatic treatment comparable to "*on-tissue*" digestion described in literature, the possibility of analyte delocalization after trypsin application was investigated. Using the piezo printer a 20 pM albumin solution was applied at a resolution of 100 μm covering 25 mm^2 homogeneously on an ITO surface and dried at room temperature. Trypsin was applied in a small rectangular shape (2 × 5 mm, 100 μm resolution) in the central area of the albumin square with the piezo printer before the sample was incubated overnight at 37 °C in an atmosphere saturated with ethanol/water (1:1, v/v). Solvents were removed on the next day by vacuum drying and MALDI matrix was applied as described again with the piezo printer. In MSI analysis the localization of albumin related peptides was investigated (Fig. 5). Albumin was identified by peptide mass fingerprinting and selected peptides further identified by PSD fragmentation. It could be demonstrated that albumin fragments were primary located in the area of previous trypsin application at a lateral resolution of 80 μm. The intensity distribution of the non-normalized data set shown in Fig. 5 reveals several signals outside the defined area, which can be related to artifacts or slight peptide diffusion. Data normalization based on the TIC levels those signals out and reveals them as noise.

After proofing the peptide localization and protein identification process of the MSI approach with albumin, proteins adsorbed to PE-UHMW were investigated. For enzymatic treatment of PE-UHMW after synovia incubation, trypsin was applied on the total polymer surface. For this, buffer adjustment turned out to be very critical as PE-UHMW is sensitive to basic pH and thus polymer hydrolysis can occur already at pH 8.5 (a pH usually adjusted for efficient tryptic digestion). The addition of Rapigest solution to enhance proteolytic degradation by denaturing proteins allowed a pH adjustment to 7.5. At this pH value sufficient enzymatic cleavage of protein was observed while polymer hydrolysis was prevented.

During the MALDI process PE-UHMW acts as an insulating material. This effect usually results in peak broadening due to increased energy transfer for ion desorption/ionization and therefore increased energy distributions as well as decreased ion extraction efficiencies. While this disadvantageous behavior is not critical for intact protein analysis as pointed out before, it clearly limits the possibilities of unambiguous

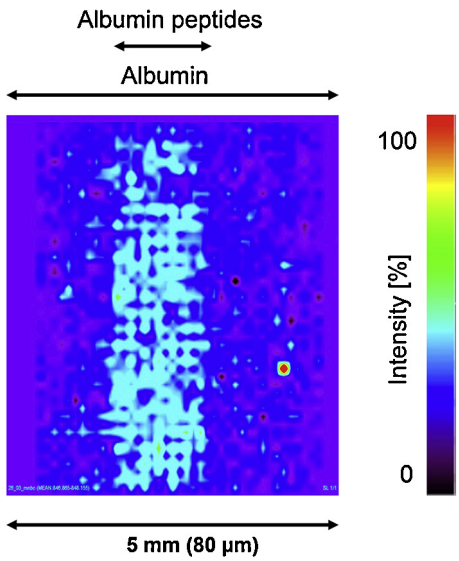


Fig. 5 – Non-normalized intensity distribution of a tryptic peptide (*m/z* 1424, [25–36] r.DAHKSEVAHRFK.d) of albumin after enzymatic digestion of an "I"-shaped area visualized with Biomap at a lateral resolution of 60 μm. The intact bovine serum albumin solution (transparent) was printed with the piezo printer in area mode on transparent ITO glass, trypsin was applied also with the piezo printer at a lateral resolution of 80 μm and the matrix (HCCA) was applied with the airbrush.

protein identification because of low absolute signal intensities and partial loss of mass resolution for the generated protonated peptides. On average 120 *m/z* signals were obtained within each mass spectrum generated for each position of the MSI experiment. Mass spectra were compared to *m/z* lists derived from *in silico* digests of already identified proteins. It was possible to correlate *m/z* values with peptides belonging to proteins present in synovia. Fig. 6 shows an exemplary profile spectrum of an "*on-polymer*" digest and the annotation of tryptic peptides for each identified protein.

For unambiguous identification peptides were fragmented by PSD experiments, however, signal intensities were only sufficient to generate neutral losses from the precursor ion and fragments related to the first few amino acids from the termini, which was not sufficient for automated database identification but confirmed protein identity when PSD spectra were interpreted manually.

Detected peptide signals were again analyzed for their distribution on the polymer samples. Similar localization preferences were found as for the respective proteins. Peptide distribution was particularly correlated with folded PE-UHMW regions, rough areas and sharp edges. Fig. 7 shows the distribution of selected peptide signals associated with albumin (*m/z* 1024, [500–508] k.CCTESLVNR.r) and apolipoprotein E (*m/z* 1104, [97–106] k.RLAVYQAGAR.e). The list of associated peptide fragments (Fig. 1) includes all high abundance proteins and the majority of low abundance proteins were also found in SDS PAGE analysis.

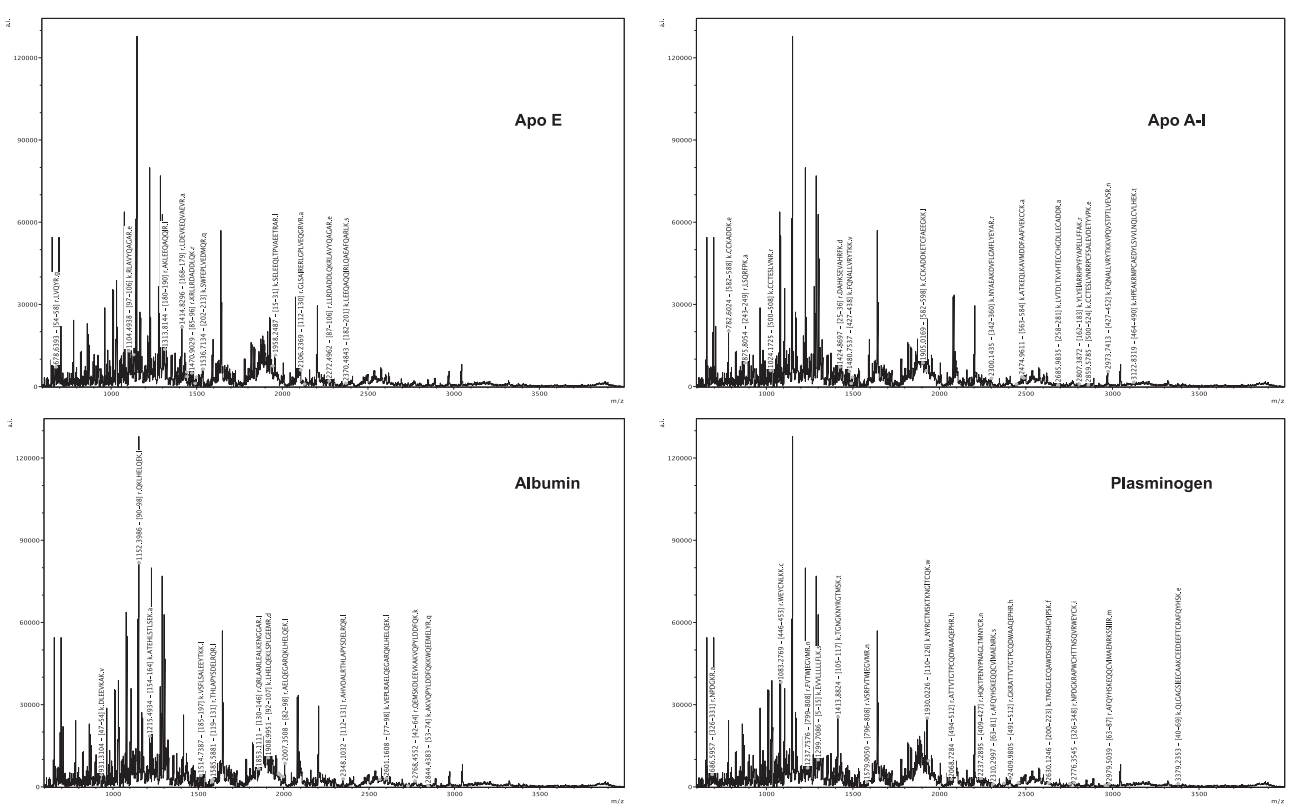


Fig. 6 – Profile mass spectrum of an "on polymer" digestion. Peptides are separately assigned to proteins according to theoretical cleavages exemplarily for apolipoprotein E, apolipoprotein A-I, human serum albumin and plasminogen.

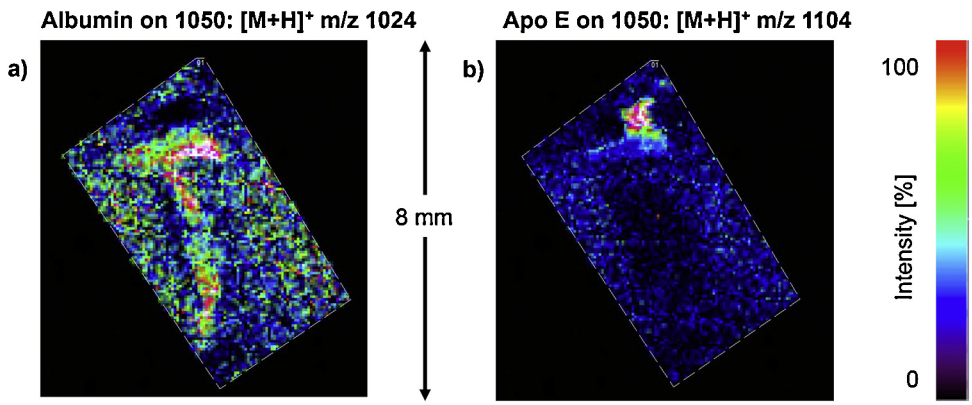


Fig. 7 – Peptide distribution after enzymatic treatment of GUR-1050 PE-UHMW (transparent) with trypsin: (a) peptide *m/z* 1024 ([500–508] k.CCTESLVNR.r) associated with albumin (b) *m/z* 1104 ([97–106] k.RLAVYQAGAR.e) associated with Apo E. Lateral resolution: 50 μm, TIC normalized data.

4. Discussion

4.1. *Protein adsorption on PE-UHMW*

Protein patterns found in SDS PAGE and MALDI-TOF analysis clearly demonstrated that synovial proteins adsorb on the polymer surface after a short time of incubation. Previous studies have been conducted only for the major synovia components in separated experiments using single molecular species like only albumin or only hyaluronic acid. Within the present study for the first time a global view on adsorbed proteins out of the complex SF was approached. Protein adsorption from synovia on differently polymer material was compared to proteins directly extracted from the very same synovia.

Protein concentrations obtained for samples containing proteins desorbed from PE-UHMW material after incubation in synovia differed significantly if two different extraction methods (ethanol and TCA precipitation) were applied. However, so far no differences for the extracted protein species were observed. The obviously low amount of detected proteins can be ascribed to the fact that SF shows very low cell count rates (<a few 1000 cells/mm^3) and consists predominantly of water. Similar to blood plasma over 85% are high abundant proteins, very good characterized regarding functionality and relevant pathways. Only major protein components of synovia were identified in this study observing the adsorption behavior on PE-UHMW in the native SF composition. The increased protein concentration observed for the extraction methods using ethanol might be related to enhanced carbohydrate and lipid extraction. These analytes are of course not detectable by SDS PAGE but may falsify protein concentration results if photometric assays are applied.

Interestingly it was observed that Vitamin E doped PE-UHMW revealed significantly higher protein concentrations according to Bradford although supposed to be less affected by protein adsorption. Vitamin E has a lipophilic structure and is therefore very likely coming off the polymer surface during extraction altering protein concentration results. SDS PAGE of proteins resulting from synovia and PE-UHMW extraction showed a very broad distribution covering a molecular mass range from 6 kDa to 204 kDa. The dominant proteins forming a layer on GUR-1050 PE-UHMW and Vitamin E doped PE-UHMW were identified to be the high abundance proteins from synovia. Intensity of protein bands shows less intense bands for Vitamin E doped material, which can be interpreted as confirmation of the fact that this material adsorbs less proteins from the biological surrounding liquid. SDS PAGE also revealed that protein size did not have an effect on adsorption as no significant intensity difference was observed for high and low molecular weight proteins. Also the degree of glycosylation of proteins seems to have no influence on preferences for PE-UHMW adsorption. Most proteins, identified on the polymer have function in signal cascades, transportation pathways or can be associated with acute phase inflammation. Latter can be explained by the fact that the SF was taken after joint revision, where inflammation is evident. However, a large number of detected proteins are associated with lipid transportation and apolipoprotein subunits were detected on all samples. Lipids are an essential part of the lubrication process and have been reported to participate in the layer formation [24]. The functionally similar apolipoproteins found on the polymer may enhance or at least support lipid layer formation.

4.2. *Protein layer formation and localization on PE-UHMW*

PE-UHMW has a very hydrophobic surface and sample preparation for MALDI profiling and MSI experiments are critical because of polymer hydrolysis. The balance between quickly evaporating solvents, neutral pH, good analyte incorporation into the matrix and sufficient protein desorption/ionization for MS analysis is important to be kept. Especially large proteins at very low concentrations are difficult to be finally detected. The chosen compromise for MALDI matrix concentration, solvent type and application method produced rather thick matrix layers, but ensured efficient extraction and analyte incorporation. The time span of matrix

application was observed to be the most critical point during the application process. Enough time for matrix crystallization between droplet depositions of each matrix layer is mandatory to ensure fine matrix crystallization also on folded or rough surface regions.

In MSI homogeneous protein patterns were observed for evenly mounted PE-UHMW samples. The fact that glycoproteins and post-translationally unmodified proteins adsorb without specific cluster formation ensures equal lubrication properties for the whole cup. For the acetabular cup *in vivo*, this means that on a new, unused acetabular PE-UHMW cup, shortly after contact with the SF, a homogeneous protein layer is formed with equal adsorption of all proteins. However, it has to be considered that the theoretical assumptions consider the interaction between SF and PE-UHMW without taking into account additional mechanical forces. In the performed *in vitro* experiments, applying SF to unused polymer samples, protein adsorption is a matter of compound concentration. And so it was of special interest that apolipoproteins, associated with a relevant function for the articulating process but not high abundant, are homogeneously distributed over the whole PE-UHMW samples already after a very short time of interaction. Vitamin E doped PE-UHMW, which is expected to be less affected by protein adsorption, showed reduced signal intensities. However, protein adsorption behavior was observed to be similar.

On both polymer materials it was interesting to see that unevenly mounted polymer slices exhibiting folded regions, rough edges or slight cuts showed preferential protein adsorption on exactly those positions. *In vivo* PE-UHMW aging goes along with polymer oxidation, which has been reported to result in very brittle surfaces [4], similar to cup irritation resulting from mechanical stress. In our experiments, morphologically altered regions were highly affected by protein adsorption According to the presented results, this means that *in vivo* stressed and damaged regions are more prone to protein adsorption. Theoretically this can imply a compensation effect due to better lubrication by the protein layer but also further damage to the material because of oxidative interaction of the protein with the polymer.

4.3. *PMF identification of proteins on PE-UHMW*

PMF and peptide sequencing after "*on-polymer*" digestion revealed two major problems: PMF identification based on theoretical peptide assignment is critical and sufficient abundant peptide PSD or CID spectra are – due to the insulating properties of PE-UHMW – difficult to obtain.

However, very complex mass spectra including the potential peptides of all enzymatically cleaved proteins from synovia were acquired. After comparing theoretical protein digests to measured *m/z* values, tentatively protein names, assigned after intact protein MSI experiments, were confirmed. Manual data interpretation of fragmented peptides confirmed those results. Again all detected peptides were found preferentially on sharp edges and folded areas.

However, PMF experiments on the polymer were also conducted to investigate the protein adsorption behavior. Peptides conserved in space after tryptic digestion and washing of the sample are considered to be strongly adsorbed on the polymer and this fact points to the possibility that these peptides are in fact those supporting the protein to stick to the material. So, investigating the adsorption behavior of single peptides can provide new insights into binding behavior and probably also diffusion behavior of proteins to modified PE-UHMW. This of course needs further investigation.

5. Conclusions

The present study shows that PE-UHMW is highly affected by protein adsorption after interaction with SF. The presented incubation experiments allowed studying whether protein adsorption is competitive if PE-UHMW materials of different types are exposed to complete SF proteomes and lipids as well as other SF components. It was observed in MSI and by gel electrophoretic experiments that all high abundance proteins in fact adsorb to PE-UHMW. The observation of homogeneous protein layers allows the conclusion that virgin PE-UHMW is completely covered with a protein layer supporting lubrication shortly after contact with SF. However, morphologically modified or rough PE-UHMW surface regions are highly affected by protein adsorption. Proteins obviously tend to adsorb on uneven surfaces. *In vivo* this behavior might induce enhanced lubrication on damaged polymer material surfaces and can result in a compensation mechanism to ensure the articulating process. Despite the expectation that Vitamin E doped UHWM-PE would be less affected, we found higher protein concentrations and comparable adsorption. Vitamin E might change the surface properties especially for preferred adsorption of either lipid or glycan species, which have not been investigated in the present study. Homogeneous layer formation has been found for both materials, which is absolutely favorable for lubrication and reduced shear stress. Nevertheless it has to be mentioned that the presented model is only a static *in vitro* model and that temperature, mechanical forces and cell stress affect protein conformation and might change *in vivo* adsorption behavior. Further investigation is necessary to evaluate protein adsorption behavior under mechanical stress to understand the interaction between PE-UHMW and SF compounds in more detail.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.euprot.2014.05.001.

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