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Isolation, characterization and quantification of polyethylene wear debris from periprosthetic tissues around total joint replacements

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Abstract

Three basic methods of isolation of polyethylene wear particles from periprosthetic tissues (alkaline, acid and enzymatic) were compared. All the three methods had to be significantly modified to obtain pure polyethylene wear particles. For isolation of wear particles the acid method was found to be the most convenient. Purity of isolated wear debris was checked by: scanning electron microscopy (SEM), energy dispersive analysis of X-rays (EDS) and infrared spectroscopy (FTIR). SEM micrographs were used as an input for automated quantitative analysis, i.e. for determination of the total number of wear particles. The reliability of our automated quantification method (called SEMq) was verified on several sets of experiments; relative errors were less than 10%. The first results, obtained by the SEMq method, indicate that the distribution of UHWMPE particles around total joint replacements is quite non-homogenous.

Keywords: UHMWPE; Isolation of wear debris; Quantification of wear particles; Total joint replacements; Arthroplasty; SEM

1. Introduction

Ultrahigh molecular weight polyethylene (UHMWPE) is still considered to be the best material for manufacture of polymer parts of total joint replacements (TJR). Excellent friction properties as well as other mechanical properties, such as tensile, impact, creep and wear behavior, are suitable for such use [1]. In spite of the high resistance of UHMWPE to wear, at every movement of the TJR a relatively large amount of microscopic wear debris is produced because of articulation of metal and polymer components [2]. A part of them, in dependence on their size, is phagocytized by macrophages in the periprosthetic tissues. This results in inflammation processes, development of granuloma, osteolysis and finally this influences the failure rates of the implants and the necessity of revision [3]. The correlation between the number of wear particles in different locations around TJRs and the extent of tissue damage in these locations necessitates fast and reliable techniques for isolation and calculation of wear particles [4].

As to the isolation of polyethylene wear particles from periprosthetic tissues, several methods have been developed and refined. These techniques were used in order to identify and characterize the size range, morphology and the number of polyethylene particles present in retrieved periprosthetic tissues [5]. Techniques of particle isolation are essentially of three types depending on the condition of hydrolysis of the organic material of the sample: hydrolysis of tissues with acid (usually HNO_3) (e.g. [6,7]), alkali (usually KOH) (e.g. [2,8–11]) and by the use of enzymes (e.g. [12]). A survey of isolation techniques could be found in the paper of Elfick et al. [13]. However, in spite of the fact that these techniques are described and published, many of them are difficult to reproduce and to use directly for polyethylene wear particle isolation. For example, the alkaline method (using 12 mol/l KOH) described by

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Bell et al. [10] is hardly reproducible and furthermore, due to unexpected vigorous reaction could even be dangerous.

As to the determination of the number of wear particles, several techniques have already been described in the literature. The techniques were listed in our previous study, in which we concluded that the existing techniques are imprecise, time consuming and/or unsuitable for processing high numbers of *in vivo* samples [4]. Techniques based on weighing (e.g. [14]) suffer from lower precision due to negligible mass of the wear debris. Also scattering based techniques, such as our own LSc method [4] or a somewhat better approach described by Elfick et al. [13], are of limited precision due to conversions of intensity, volume and number distributions. The other techniques do not take into account the smallest particles with sizes below 0.5 μm [6,15] or they determine only the overall wear [16,17]. In this work we introduce yet another technique, based on automated image analysis of electron microphotographs of isolated polyethylene (PE) wear particles on polycarbonate (PC) membranes.

This study deals with two main problems: (i) optimization of methods for isolation of polyethylene wear debris from periprosthetic tissues and (ii) introduction of a reliable technique for determination of total amount of wear particles around TJRs. Possible drawbacks of the isolation techniques already described in the literature are discussed. The first results of our newly introduced technique, confirming that the distribution of wear particles around TJRs is quite non-homogeneous, are shown.

2. Materials and methods

Periprosthetic tissues were obtained from various, well-defined zones around total joint replacements during revision surgery [4]. Collected samples were freeze-dried and kept at laboratory temperature before use.

2.1. Purification of chemicals

Distilled water, 0.015 mol/l $CaCl_2$, enzyme solutions and isopropyl alcohol (iPrOH) were purified before use by successive filtration through 10 and 0.1 μm polycarbonate (PC) Cyclopore membranes (Whatman, UK). More aggressive chemicals, such as 65% HNO_3, 6 mol/l HCl, 5 mol/l NaOH and 12 mol/l KOH were filtered through 10 and 0.1 μm Teflon (PTFE) membranes (Millipore, Ireland).

2.2. Delipidation of samples

Typically, 0.3 g of freeze-dried sample was cut into small pieces and extracted twice with 10 ml of a chloroform/methanol mixture (2:1, v/v) for 12 h. The solvents were decanted. After the second decantation the samples were dried with a stream of filtered air and by heating at 60 °C for 2 h.

2.3. Alkaline hydrolysis

The delipidated sample (obtained from 0.3 g of freeze-dried sample) was heated with 5 ml of 12 mol/l KOH [10] at 60 °C for 3 days with occasional shaking, followed by neutralization with 6 mol/l HCl (ca. 10 ml). The whole sample (ca. 15 ml) was centrifuged at 2000 × *g* for 10 min. The sediment and the lower layer of supernatant (ca. 13 ml) was removed by aspiration. The removal of supernatant was justified by the fact that only negligible amounts of polyethylene particles were obtained after filtration of the lower layers of the supernatants through the 0.1 μm membrane. The remaining 2 ml of the sample was mixed with 5 ml of distilled water, centrifuged at 500 × *g* for 1 min and the lower 5 ml of the sample were again removed by aspiration; this washing with H_2O was repeated twice. During the development of the method, the collection of polyethylene particles from the upper layer of supernatant after centrifugation for a longer time and at higher *g* was tried. The conditions finally used (500 × *g* for 1 min) were found sufficient as the lower layers of the supernatant gave negligible amount of particles after the filtration through the 0.1 μm membrane. The resulting 2 ml of the sample were used for further purification.

2.4. Acid hydrolysis

The delipidated sample (obtained from 0.3 g of freeze-dried sample) was hydrolyzed with 5 ml of 65% HNO_3 for 24 h at room temperature. Only the upper 2 ml layer was used for further isolation, remaining lower part was removed by aspiration and discarded. This upper layer was twice washed with 5 ml of fresh 65% HNO_3 and twice with 5 ml of distilled water. The washing procedure was analogous to that described in previous paragraph: 5 ml of solvent (HNO_3 or H_2O) was added, the sample was centrifuged (500 × *g*, 1 min) and the lower 5 ml were removed by aspiration. The resulting washed 2 ml layer was neutralized with 12 mol/l KOH and twice washed with water in the same way as described above. The resulting 2 ml of the sample was used for further purification.

2.5. Enzymatic hydrolysis

The delipidated sample (obtained from 0.3 g of freeze-dried sample) was homogenized in 5 ml of 0.015 mol/l $CaCl_2$ with an X620 CAT (Germany) homogenizer, pH was adjusted to 8.5 with 5 mol/l NaOH solution and pronase from *Streptomyces griseus* (Sigma, USA) (5 mg/ml) in 3 ml of 0.015 mol/l $CaCl_2$ was added. The pH value was readjusted to 8.5 and the reaction mixture was incubated for 48 h at 37 °C. Then the same amount of pronase was added once more (due to autodigestion of proteolytic enzyme—pronase), pH was readjusted to 8.5 and the digestion was continued at 37 °C for another 24 h. The reaction mixture was then heated for 10 min in a boiling water bath. After cooling pH was adjusted to 7.5 with 6 mol/l HCl and collagenase from *Clostridium histolyticum* (Sigma, USA) (5 mg/ml) in 3 ml of water was added. The mixture was incubated at 37 °C for 72 h. Then the second portion of the same collagenase (Sigma, USA) solution was added and the reaction mixture was incubated for additional 24 h at 37 °C. Reaction mixture was then centrifuged at 1000 × *g* for 5 min and 2 ml of the upper layer of the supernatant was twice washed with 5 ml of water, in the

same way as described in the previous two paragraphs, i.e. by centrifugation (500 × *g*, 1 min) and aspiration of lower 5 ml of the solution. The final washed 2 ml of the sample was used for further purification.

2.6. Separation of wear debris by filtration in aqueous iPrOH solution

Each washed 2 ml wear debris suspension obtained by alkaline, acid or enzymatic hydrolyses was mixed with 4 ml of iPrOH and filtered through 10 μm polycarbonate Cyclopore membrane (Whatman, UK). The filtrates were finally filtered through an 0.1 μm membrane of the same provenience. These membranes with adhered polyethylene wear particles were used for checking of purity and characterization of the particles.

2.7. Scanning electron microscopy (SEM)

The polycarbonate (PC) membranes with polyethylene (PE) wear particles were sputtered with platinum (vacuum sputter coater, Balzers). A layer of thickness 10 nm was found necessary to prevent sample damage in the microscope. The samples were observed in SEM microscope Vega TS 5130 (Tescan, Czech Republic), using secondary electron detector, accelerating voltage 30 kV and magnifications 4000× or 5000×.

2.8. Energy dispersive analysis of X-rays (EDS)

The same samples as those used for SEM were observed in the microscope XL 30 ESEM (FEI, Czech Republic) equipped with an EDS detector (EDAX, USA). SEM images were obtained at accelerating voltage 30 kV. EDS point analyses were performed with all kinds of particles and background. The peak corresponding to thin platinum layer was ignored. Not fewer than 10 analyses of each investigated sample were made.

2.9. Infrared spectroscopy (FTIR)

FTIR spectra were recorded with an IFS-55 spectrometer (Bruker, Germany) equipped with MCT detector (256 scan/spectrum, resolution 4 cm^{-1}). Selected PC membranes with PE particles were measured before sputtering with platinum and investigation by SEM and EDS. The measurement was performed in two ways: (i) several locations on each investigated membrane were measured by ATR technique using a Golden GateTM Heated Diamond ATR Top-Plate (Specac Ltd.) and (ii) the whole membrane was measured in transmission mode.

2.10. SEMq: automated quantification of wear particles

The automated quantification of wear particles was made by image analysis of SEM micrographs. All the micrographs came from the same microscope (Vega TS 5130), had the same size (768 × 768 pixels), approximately the same brightness and contrast (i.e. as similar histograms as possible) and the same magnification (either 4000× or 5000× for all images in a series of the samples). The close similarity of the micrographs was quite important because the images were used as an input to an automated image analysis routine, which was a macro created in software Lucia (LIM, Czech Republic). Briefly, the macro works in the following way: (i) it asks the user to insert the name of an input image, (ii) reads the input image, (iii) asks the user to estimate a threshold,[1] (iv) automatically performs a set of binary operations,[2] which separate the objects (in our case PE particles) from the background (PC membrane), (v) calculates the total area of the image and the total area covered by the objects and (vi) outputs the fraction of the area (*AreaFraction*, AF) covered by the particles. The AF value was proved to be very well proportional to the total amount of the wear particles in the sample. If all the analyzed samples contain particles with equal average sizes, if all the experiments are performed in the exactly same way and if AF if the particles do not overlap, the AF is even directly proportional to relative number of particles, *N*:

$$N = \mathrm{AF}. \tag{1}$$

If the analyzed samples contain particles with different average sizes, the relative number of particles is determined as follows: (i) AF is determined as described above (ii) average particle area (AP) in each zone is determined by independent, standard image analysis (e.g. [18–22]) and (iii) AF is divided by AP. If the mass of the sample used for the analysis (MS), total area of the membrane (AM) and fraction of suspension filtered through the membrane (FS) are the same, the value of AF/AP represents the relative number of particles (*N*) in a given zone:

$$N = \frac{\mathrm{AF}}{\mathrm{AP}}. \tag{2}$$

If the above parameters (MS, AM, FS) differ from sample to sample, the results can be normalized by means of the following formula:

$$N = \frac{1}{\mathrm{MS}} \times \frac{1}{\mathrm{FS}} \times \mathrm{AM} \times \frac{\mathrm{AF}}{\mathrm{AP}}. \tag{3}$$

If all the parameters in Eq. (3) have compatible units, *N* represents the absolute number of wear particles per 1 g of dried sample. The whole procedure was called SEMq (i.e. quantitative analysis of SEM micrographs). The principle of SEMq is schematically shown in Fig. 1; more details can be obtained directly from the authors.

[1] Threshold is a value between 0 and 255. In grayscale images, completely black pixels have intensity = 0, completely white pixels have intensity = 255. If the threshold equals 100, all pixels with intensity ≥100 turn white and all pixels with intensity <100 turn black. In our macro, the threshold is set interactively, i.e. the user just moves the mouse and sees the changes in the image. After setting the threshold the user obtains black-and-white image, called binary image.

[2] Binary operations work with the black-and-white, binary images. For example the binary operation called *Erosion* removes pixels from the edges of all objects. More information can be found in any manual to image analysis software.

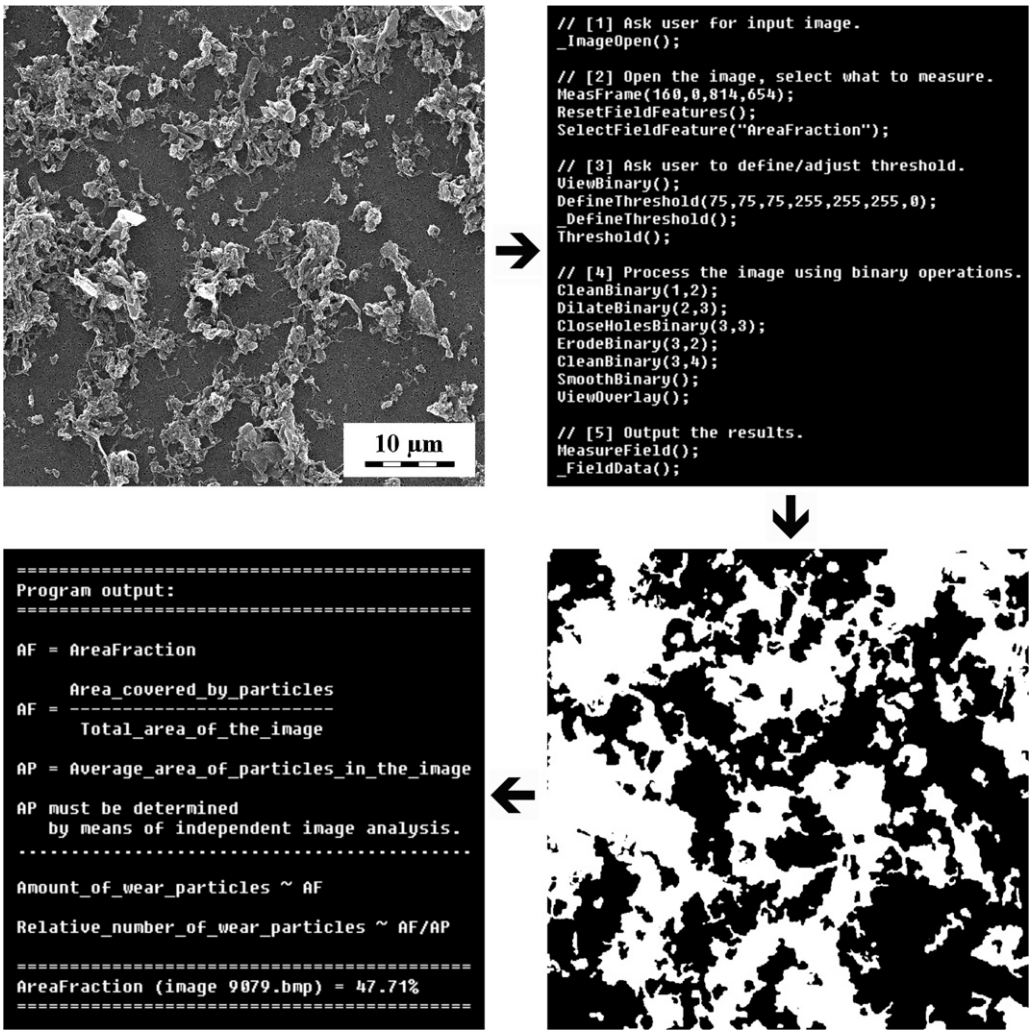

Fig. 1. SEMq method: scheme of the automated quantification procedure. Small black arrows show the course of the procedure. Top left image: input SEM micrograph; top right: the macro converting a SEM micrograph to a binary image and determining *AreaFraction*; bottom right: binary image, i.e. SEM image after processing with the macro; bottom left: output of the program giving the area fraction of PE particles.

3. Results

3.1. Isolation of PE wear particles

All three tested methods of isolation of PE wear particles yielded, after some modifications, more-or-less the same results as for the purity of the particles. Some of the samples processed by enzymatic hydrolysis, however, contained certain amounts of bone fragments. Considering all aspects, such as the time needed for isolation, cost of chemicals and final purity of the isolated particles, the acid method was found the most convenient.

3.2. Characterization of isolated PE wear particles

The isolated samples, i.e. the PE particles on PC membranes coming from all three isolation procedures described above, were characterized by three independent methods, whose results were used for perfecting all the three isolation procedures. In the first step, the SEM microphotographs were inspected and compared with those found in the literature (e.g. [18–22]). Some micrographs showed not-well-purified samples, a few micrographs showed a small amount of inorganic microcrystals indicating insufficient washing and in some micrographs even bacteria were observed. All these impurities were successively removed by modifying the isolation procedures. In the second step, when the samples seemed pure according to the SEM micrographs, the samples were analysed in SEM microscope with an EDS detector. Except for the three elements, which were always present (C from PE and PC, O from PC and Pt from sputtering), also the elements indicating bone fragments (P, Ca), proteins (N, S, P) and phospholipids (P) were searched for. In the end all samples were found pure with the exception of enzymatic isolation, where some amounts of P and Ca were found, suggesting that the samples contain also microscopic bone fragments. This could not be improved because the enzymes, which were used in this study, do not digest bones. In the third step, the samples, whose SEM micrographs and EDS analyses indicated only pure PE particles, were investigated by FTIR. Spectra collected in the transmission mode were dominated by peaks from PC membrane as it was approximately one

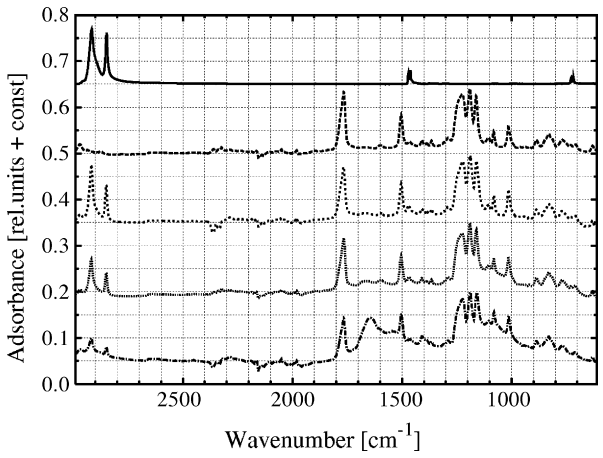

Fig. 2. FTIR spectra: ATR measurement, comparison of isolation techniques. Spectra from top to bottom: PE from catalogue (full line), PC membrane (dashed line), PC membrane + PE particles after HNO_3 isolation (dotted line), PC membrane + PE particles after KOH isolation (finely dotted line) and PC membrane + PE particles after enzymatic isolation (dash-and-dot line).

order of magnitude thicker in comparison with PE particles; except for peaks corresponding to PC, only PE peaks could be identified, which suggested that the amount of impurities was negligible. Selected spectra collected in the ATR mode are shown in Fig. 2; at least two spectra from two different locations were measured for each membrane. In ATR, the intensity of PC peaks is comparable with the intensity of PE peaks, namely the two at 2919 and 2848 cm^{-1}, which represent antisymmetric and symmetric stretching CH_2 vibration, respectively. Moreover, in the area between 1900 and 1500 cm^{-1}, the spectrum contains some peaks corresponding to impurities, as is easily seen by comparing the spectra of pure PE and pure PC on one hand with the spectra of isolated PE particles on PC membranes on the other hand. The comparison also proves that HNO_3-based isolation yields the best results: the whole spectrum of PE particles on PC membrane, except for the two intensive PE peaks around 2900 cm^{-1}, is almost identical with the spectrum of pure PC membrane, indicating that the HNO_3-isolated particles are almost pure PE.

3.3. Automated quantification of particles by SEMq method

The SEMq method was introduced to prove our assumption that the distribution of numbers of wear particles in different locations around particular TJR is quite non-homogeneous. The quantification method based on light scattering, called LSc, which we used in our previous work [4], suggested that the assumption is correct but the results are not found fully reliable due to agglomeration effects and numerous re-calculations, which are inherent to light scattering. From this point of view, the main advantage of SEMq is the fact that the method is very intuitive and clear: the number of particles is simply estimated from SEM micrographs using Eq. (3) and the reliability of the results can be checked by visual inspection of corresponding micrographs.

In the first step, it was necessary to prepare model samples to check whether the SEMq method works. Three different model series of samples were prepared. One series involves a set of PC membranes covered with a certain amount of *in vivo* PE particles. All membranes in a given series contained the *in vivo* PE particles with the same size distribution, because all the particles in a given model series came from the same source. As the SEMq technique yields the results on relative scale, it is quite sufficient to guarantee that the concentration of PE particles on PC membranes grows in a defined way. The increase in concentration of PE particles was achieved as follows: the suspension of isolated *in vivo* PE particles in iPrOH was divided into several parts, e.g. 2, 4, 6 and 8 ml, and each part was filtered through one membrane. In such a case, the concentration of PE particles on the membranes exhibited the ratio 1:2:3:4. The three model series of samples (denoted as S1, S2 and S3) are summarized in Table 1. SEM micrographs corresponding to the smallest series S1 are shown in Fig. 3. Each micrograph represents a random location on a membrane. In series S1, four random locations, each from one quadrant of the membrane, were saved as SEM micrographs. In series S2 and S3, eight random locations per membrane (i.e. two from each quadrant of the membrane) were recorded and processed.

In the second step, the micrographs from model series were processed by SEMq and/or manually to obtain binary images and, consequently, to calculate *AreaFractions* (AF) as shown schematically in Fig. 1. As all the samples within the model series contained the particles with the same size distributions, the relative numbers of particles and *AreaFractions* are identical (Eq. (1)). The values of AF for all three series are shown in Fig. 4: the increase in AF with growing PE concentration is clearly visible. The reproducibility of SEMq results is illustrated in Fig. 5, which contains comparison of three different image processing techniques. The first image processing was completely manual, using binary editor in LUCIA software. The second image processing was based on a simple fully automatic macro with fixed threshold and the third image processing employed the

Table 1
Model series, which were used for checking the SEMq method

Series name	Suspension volume (ml)	No. of samples	Concentration on membranes (i.e. ml of suspension filtered through a membrane)
S1	25	3	2 ml, 4 ml, 6 ml, 8 ml
S2	20	4	0.125 ml, 0.25 ml, 0.5 ml, 1 ml, 2 ml, 4 ml, 8 ml
S3	40	4	3 ml, 4 ml, 5 ml, 6 ml, 7 ml, 8 ml

PE particles from several samples (column no. of samples) were acid-isolated and obtained as a suspension of pure PE particles in iPrOH (column suspension volume). The model series (column series name) were obtained as a set of 0.1 μm PC membranes, though which selected amounts of the suspension were filtered (column concentration on membranes).

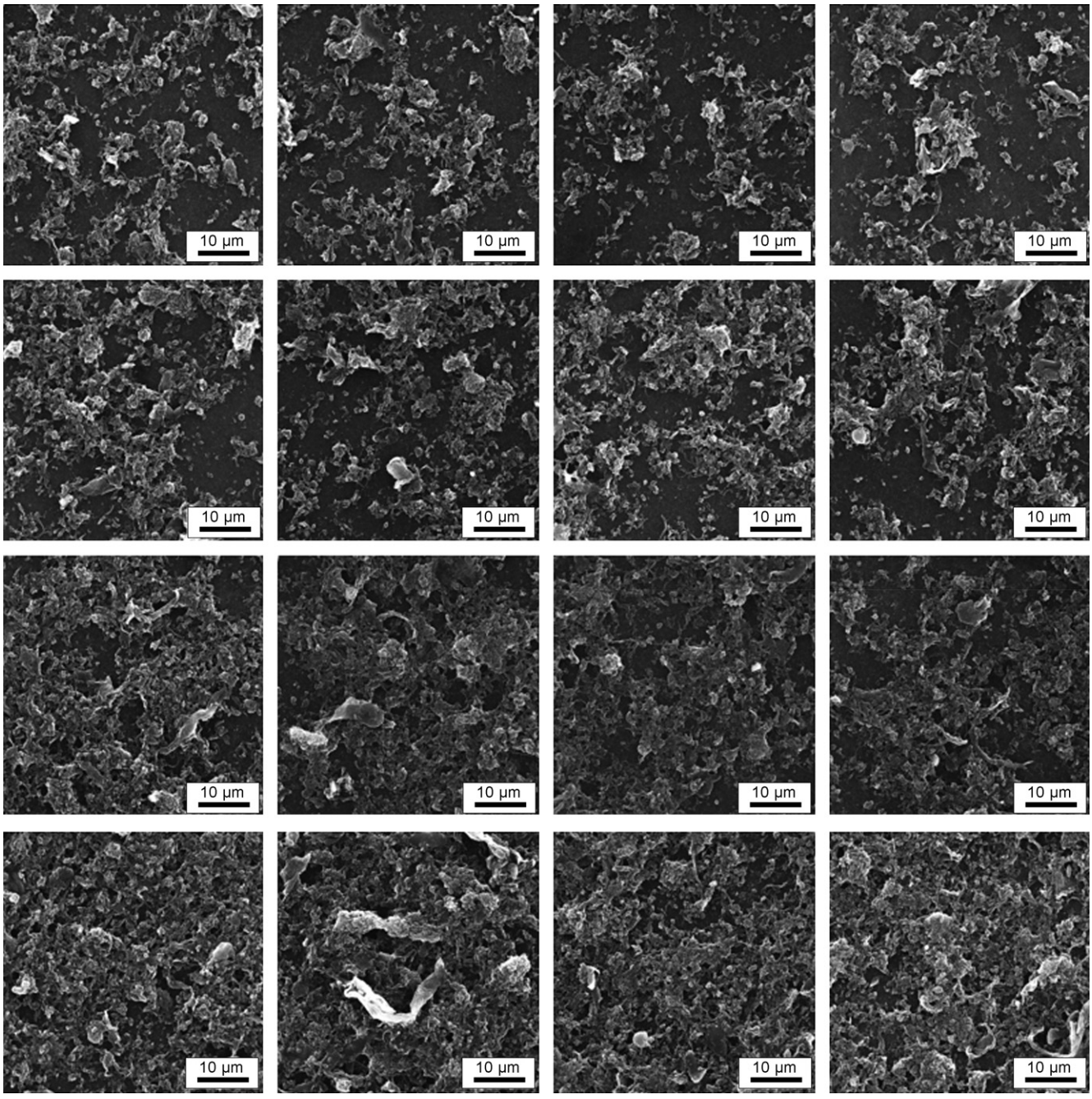

Fig. 3. SEM micrographs of model series S1 showing HNO_3-isolated PE particles on 0.1 μm PC membranes. The first top row displays PE particles coming from 2 ml of suspension. The second, third and fourth row show 4, 6 and 8 ml of the same PE suspension filtered through PC membrane, respectively. The locations on the PC membranes were selected randomly.

macro shown in Fig. 1 that was actually used in SEMq. As all three image processing techniques in Fig. 5, in particular manual and SEMq, yielded almost identical results, the method is reproducible and independent of which user or program sets the threshold and estimates the key parameter, *AreaFraction*.

In the third step, the results from model series were used to check the linearity and estimate the precision of SEMq. Firstly, the *AreaFraction* should be a linear function of PE particles concentration, which is evidenced in Fig. 6. Secondly, the linear relationship between *AreaFraction* and PE concentration is limited because the *AreaFraction* cannot run beyond 100% as illustrated in Fig. 6a: if the *AreaFraction* values make more than ca 70%, the linearity vanishes. The explanation is that more-and-more wear particles are in several layers if PE concentration increases. Fig. 6 does not show series S1 because the PE concentrations were quite high and, as a result, the values of *AreaFractions* were in too-high regions. Thirdly, the linear regression lines in Fig. 6 should converge to zero with decreasing PE concentration, but this is not fulfilled entirely as the regression lines cross the *y*-axis slightly above zero in both cases. Evidently, the macro in SEMq somewhat overestimates the *AreaFraction*, which seems to be a penalty for automation.

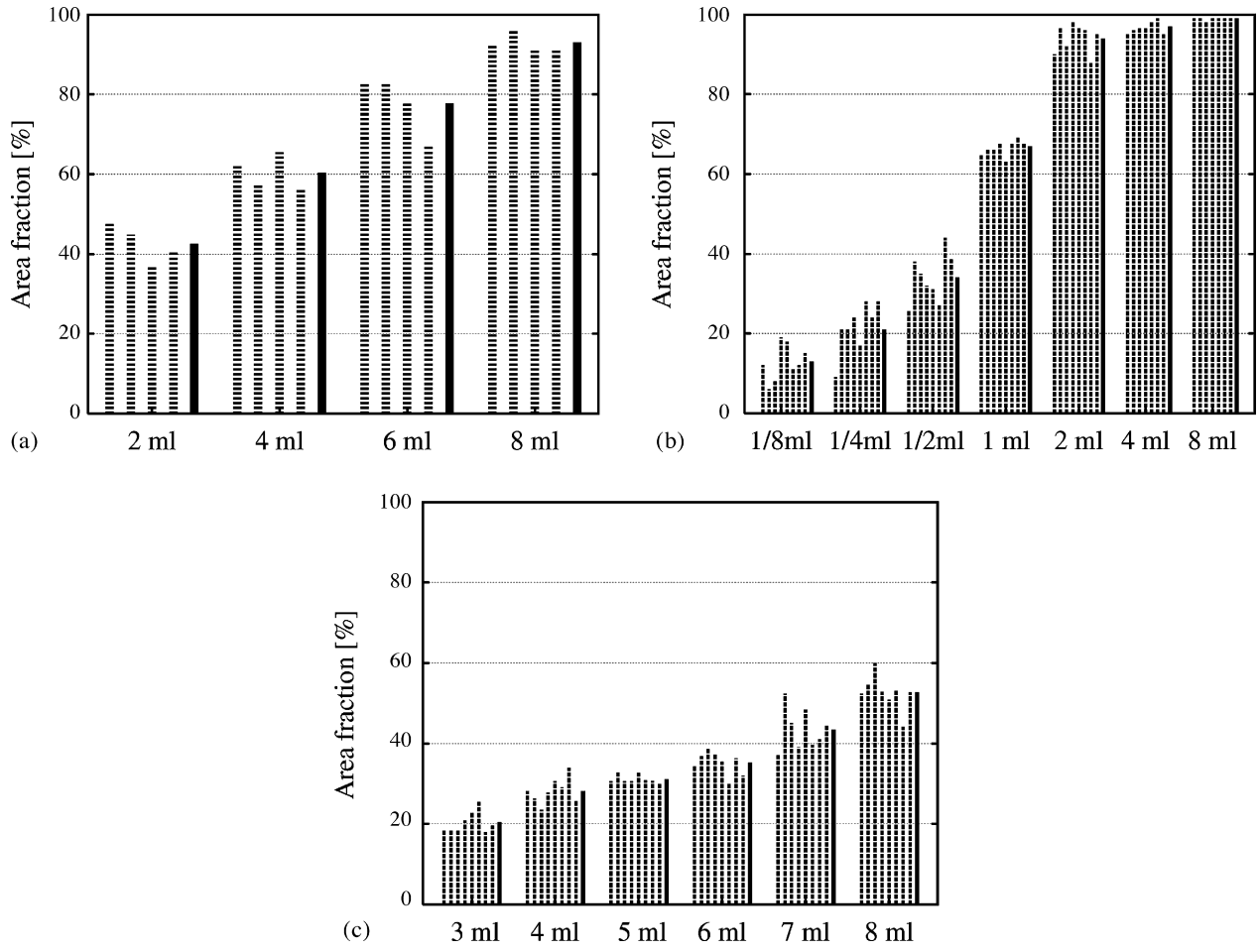

Fig. 4. SEMq method: the results of the automated quantification procedure for series (a) S1, (b) S2 and (c) S3. *x*-Axis: increasing concentration of PE-particles. *y*-Axis: micrograph area covered by the particles. Dashed lines represent individual measurements; full lines are the arithmetic averages of each set.

Table 2
Estimation of the precision of SEMq method, model series S2

Concentration (ml)	Area fraction (exp.) (%)	Area Fraction (calc.) (%)	Absolute difference (%)	Relative difference (%)
0.125	13.19	13.22	0.03	0.21
0.25	21.92	20.84	1.08	4.93
0.5	34.51	36.08	1.57	4.56
1	67.09	66.57	0.52	0.77

Area fraction (exp.) and Area Fraction (calc.) are values of Area fraction from experiment and calculation based on regression curve $y = ax + b$, respectively. Relative_difference is the error calculated as Absolute_difference/Area_fraction_(exp) × 100.

Table 3
Estimation of the precision of SEMq method, model series S3

Concentration (ml)	Area fraction (exp.) (%)	Area Fraction (calc.) (%)	Absolute difference (%)	Relative difference (%)
3	20.34	20.05	0.29	1.42
4	28.18	26.09	2.09	7.40
5	31.08	32.14	1.06	3.40
6	35.26	38.18	2.92	8.28
7	43.44	44.22	0.78	1.80
8	52.65	50.27	2.38	4.53

Area fraction (exp.) and Area Fraction (calc.) are values of Area fraction from experiment and calculation based on regression curve $y = ax + b$, respectively. Relative difference is the error calculated as Absolute_difference/Area_fraction_(exp.) × 100.

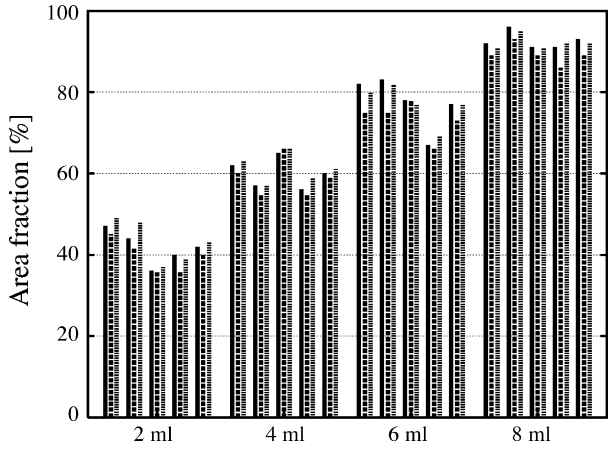

Fig. 5. SEMq method: comparison of various image analysis techniques for samples from model series S1. *x*-Axis: increasing amount of the suspension of PE wear particles. *y*-Axis: area on SEM micrographs, covered by the particles. Each column represents one SEM micrograph; the first four triplets of column in the groups are individual measurements, the last triplet of columns in each group is the average. Black columns stand for the values from manual image processing; dotted and striped columns stand for the values from fixed-threshold macro and macro used in SEMq, respectively.

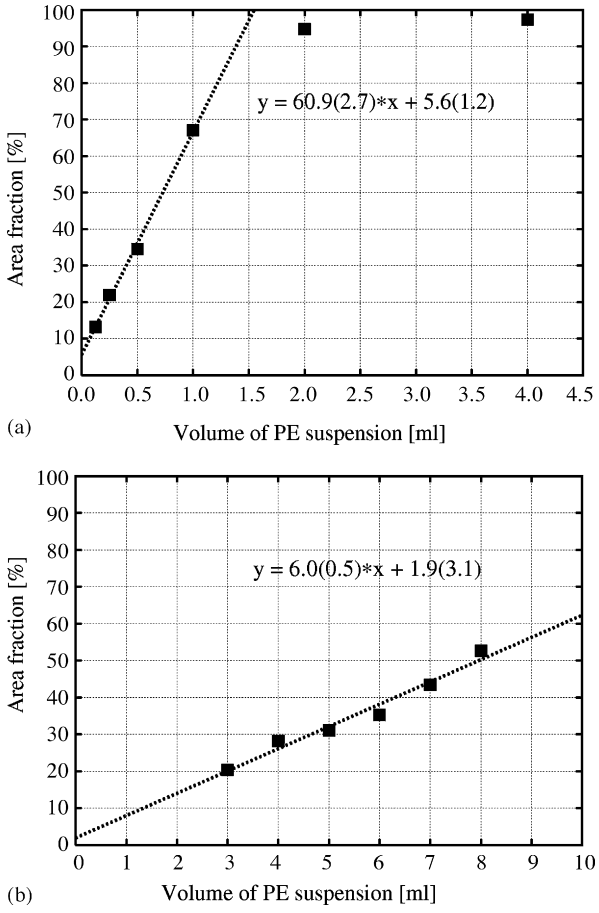

Fig. 6. SEMq method: linear increase in the area covered by particles with increasing concentration of the particles for series (a) S2 and (b) S3. Black squares are experimental *AreaFractions*, dotted lines represent linear regressions. In (a) the linear regression was calculated using just the first four points.

Finally, twice higher concentration should result in twice higher *AreaFraction*. This is not fulfilled absolutely, probably for two reasons: (i) the PE particles tend to agglomerate and lie in several layers as the concentration increases and (ii) as discussed above, the macro seems to slightly overestimate the *AreaFraction*, which introduces an additive constant to each *AreaFraction* value. The precision of the SEMq method was estimated as the maximum difference between linear regression curves and experimental data (Tables 2 and 3). The highest difference found was 8.3% of the measured *AreaFraction* value. Hence it can be concluded that the SEMq estimates numbers of wear particles with a precision better than 10%, being aware of the fact that the error increases at very low (*AreaFraction* < 10%) and very high PE concentrations (*AreaFraction* > 70%).

In the last step, the SEMq method was applied to several real samples (Table 4). In this case, the experimental conditions were the same for all samples, but some samples contained particles with different sizes (Table 5) and, as a result, the relative numbers of particles had to be calculated according to Eq. (2) as documented below. The results are summarized in Fig. 7; they prove that the numbers of particles found in various zones of one patient may differ from each other as much as one order of magnitude. Fig. 7a shows the values of AF, which would be directly proportional to the numbers of wear particles on condition that the average particle sizes in all zones would be equal (Eq. (1)). This is, however, not the case as documented in Table 5; average particle diameters (ED) and areas (AP) somewhat differ not only for the individual cases, but also for the individual zones. Therefore, the true relative numbers of wear particles were calculated

Table 4
Summary of studied patients and total joint replacements

Case	Sex	Birth	Implant type	Implant duration (years)	Original disease	No. of zones	Images per zone
K1	M	1933	PFC J + J	8	RA	6	8
K2	F	1946	DePuy[a]	10	OA	6	8
K3	M	1928	PFC	9	OA	4	8
H1	F	1969	WM[b]	9	OA	4	8
H2	F	1933	Poldi/LOR[c]	17	OA	4	8
H3	M	1935	Balgrist/CF30	8	OA	1	8

Case: three cases of total knee replacement (K1–K3), three cases of total hip replacement (H1–H3). Implant type:
[a] DePuy LCS menisceal bearings.
[b] Walter-Motorlet, Czech Republic.
[c] From 1988 Poldi implant, after revision in 1999: LOR implant (Sulzer). Original disease—OA: osteoarthritis; RA: rheumatoid arthritis. Number of zones: the number of locations around joint replacement, from which the samples were taken; the total number of processed images was: 25 (zones) × 8 (images/zone) = 200.

Table 5
Summary of average particle sizes, areas and numbers

Case/zone	ED (μm)	AP (μm^2)	AF (%)	N (rel. units)
K1/1	0.59 ± 0.36	0.37	36	96
K1/2	0.50 ± 0.31	0.27	11	40
K1/3	0.55 ± 0.37	0.34	20	58
K1/4	0.56 ± 0.36	0.35	43	123
K1/5	0.54 ± 0.35	0.33	26	80
K1/6	0.50 ± 0.34	0.29	24	84
K2/1	0.52 ± 0.41	0.35	27	78
K2/2	0.57 ± 0.40	0.38	8	21
K2/3	0.53 ± 0.35	0.32	13	41
K2/4	0.49 ± 0.42	0.32	11	34
K2/5	0.51 ± 0.37	0.31	8	26
K2/6	0.50 ± 0.40	0.32	19	60
K3/1	0.50 ± 0.44	0.35	41	118
K3/2	0.45 ± 0.41	0.28	46	162
K3/3	0.49 ± 0.40	0.31	56	181
K3/4	0.51 ± 0.47	0.38	09	24
H1/1	0.69 ± 0.46	0.54	35	65
H1/2	0.69 ± 0.45	0.53	41	78
H1/3	0.64 ± 0.47	0.50	24	48
H1/4	0.70 ± 0.40	0.51	39	77
H2/1	0.30 ± 0.21	0.10	9	86
H2/2	0.32 ± 0.16	0.10	27	267
H2/3	0.32 ± 0.24	0.12	5	40
H2/4	0.31 ± 0.15	0.09	4	44
H3/1	0.48 ± 0.36	0.28	39	138

Case: defined in Table 4. Zone: zones were just numbered consecutively. ED: average equivalent diameter of the particles in given zone, determined from image analysis, given as average ± standard deviation. AP: average area of the particles in given zone, determined from image analysis. AF: AreaFraction = area covered by PE particles on PC membrane. N: relative number of particles, calculated as AF/AP, i.e. AreaFraction divided by average area of the particles in the corresponding zone.

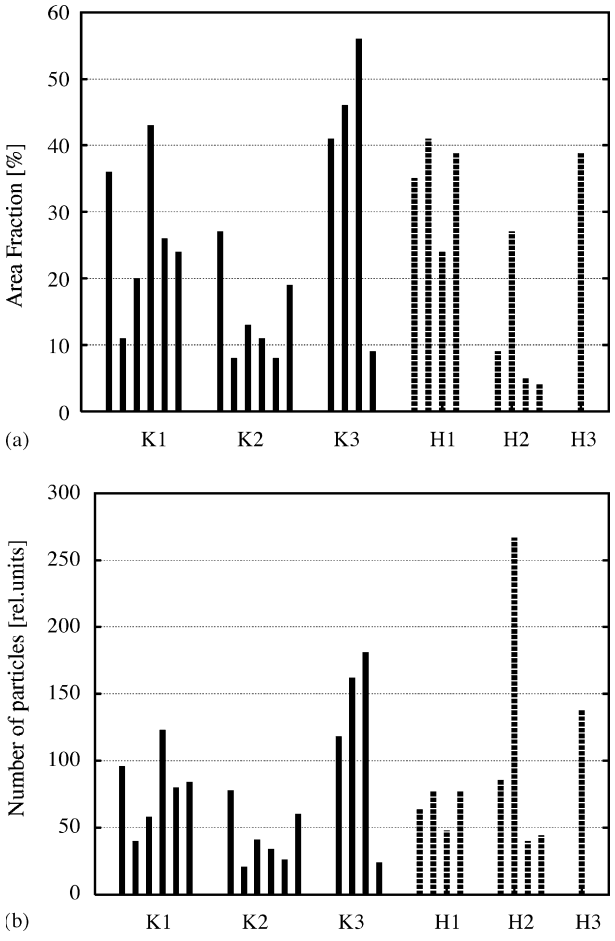

Fig. 7. SEMq method: the first results achieved on real samples—(a) *AreaFractions* and (b) relative number of particles found in various locations around TJR. Each column represents one location in particular TJR. K1, K2, K3 and H1, H2, H3 denote TKR and THR from patients 1 to 6, respectively.

as AF/AP (Eq. (2)) and shown in Fig. 7b. We may note three interesting facts: (i) the average size of the particles seems to differ more among the patients than among the zones within each patient, (ii) the correction for AP did not influence the results strongly because the order of zones according to the numbers of particles remained almost the same with only two exceptions and (iii) after recalculation it showed that the highest number of wear particles was found in one zone of case H2, which was the implant with the highest lifetime (Table 4). As soon as more results are available correlation between the numbers of particles in particular zones and the extent of tissue damage in these zones will be evaluated.

4. Discussion

Very similar results were obtained by the three methods described above (alkaline, acid and enzymatic) as regards to purity of isolated wear debris. However, these methods significantly differed in time consumption and other factors.

The alkaline method, described by Bell et al. [10], is hardly reproducible because the used polycarbonate Cyclopore membranes (Whatman, UK) are attacked by 12 mol/l KOH and this solution is not neutralized before filtration according to Ref. [10]. Furthermore, delipidation mixture (chloroform/methanol) vigorously reacts with 12 mol/l KOH ($CHCl_3 + 3\ KOH \rightarrow HCOOH + 3\ KCl + H_2O$). Also, the five-day heating during hydrolysis is tedious. The modification of the alkaline method described by Mabrey et al. [11] shows drawbacks in hydrolysis: 1 h at 65 °C in 5 mol/l NaOH is insufficient. Purification in a saccharose density gradient requires good centrifuge and is also tedious. Similar problems could be found in other modifications of the alkaline method [9], which forced us to introduce our own improvements of the method as described in Section 2.

The enzymatic method has a general disadvantage of the cost of enzymes and long hydrolysis times. We selected a combination of two enzymes pronase and collagenase instead of papain [12], because it was difficult to complete the hydrolysis of tissues with papain only. Bone fragments were observed macroscopically in nearly all tissue samples, but only the enzymatic method was able to isolate them together with polyethylene particles, which can be advantageous for studies dealing also with non-polyethylene debris in periprosthetic tissues. In enzymatic method, the bone fragments were frequently found in precipitates of hydrolysis mixtures after centrifugation. Also other odd particles and/or objects, not observed in the alkaline and acid method, were observed (results not shown here).

In our hands the acid method, whose principle was described by Margevicius et al. [6], and which was modified by us, seemed to be the most convenient. It does not require heating and, moreover, the hydrolysis time is relatively short. Therefore, the method is suitable for processing of large numbers of samples.

During testing the three above mentioned methods we found that preliminary filtration (prefiltration) of all solutions is necessary to obtain pure polyethylene debris. This prefiltration is used in the published methods only exceptionally [6]. The advantage of the use of aqueous iPrOH for final separation of polyethylene wear debris is in uniform spreading of the debris on the membrane. Otherwise clusters of wear particles could be formed.

The existing methods for determination of the amount of wear particles have already been discussed in the introduction section. Nevertheless, it is worth re-emphasizing that the wear particles quantification, i.e. determining their number, not just their morphology, is not easy. As a result, there are many publications dealing with morphology of the particles (e.g. [18–21] and references therein) but just a few papers on quantification of wear particles in various locations around TJR are available [13]. However, it is true that several wear particles quantification techniques have been described (e.g. [4] and references therein), and even several studies employing analysis of SEM micrographs to determine the amount of wear particles have been published (e.g. [2,7,14,22,23]). The originality of the SEMq technique consists in several facts: firstly, the most tedious part of the SEMq method, the image processing, was successfully automated (Fig. 1). Secondly, it was proved by means of three model series of samples (Table 1) that the combination of our HNO_3-isolation procedure and SEMq yields consistent, reasonable, precise and reproducible results (Figs. 3–6). Thirdly, the method is based on the total area covered by particles, not on calculating the single particles, which means that the results are almost independent of agglomeration. And finally, the present work is one of the few studies trying to determine the numbers of wear particles in various locations around TJR. The current results suggest that the distribution of wear particles around TJR is quite non-homogeneous (Fig. 7). This conclusion accords with our previous study [4], which was based on the LSc scattering technique of particle quantification, and supplements the study of Elfick et al. [13], who used a similar method based on light scattering but came to the conclusion that the numbers of particles in different zones vary little.

The SEMq method has two characteristic features. The first feature and important advantage consists in that the method is straightforward and simple. The results are understood intuitively and are easy to check visually because the area covered by PE particles is proportional to the number of the particles (Fig. 2). The correction for different particle sizes in various zones is important, but does not change the results significantly as far as the relative numbers of wear particles within individual patients are concerned (cf. Fig. 7a and b). Therefore, the SEMq method can be used for verification of faster, but not so intuitive methods, such as LSc [4]. A second feature of the SEMq method is the fact that SEMq was developed primarily to *compare* the numbers of particles in various zones around TJR, not to determine their absolute numbers. That is why the method is very reliable in determination of *relative numbers* of wear particles, especially if the particles are of the same size (Eq. (1), Figs. 3–6). Even if the particles differ in size (Table 5), the relative numbers of wear particles are easily determined (Fig. 7), because the key parameter, AP (Eq. (2)), is determined by parallel image analysis, which can be performed in the same way for all investigated samples. However, if we want to calculate *absolute numbers* of wear particles, the result will strongly depend on the precision of the second, parallel image analysis, namely on the evaluation of the smallest particles. In our future studies, we are going to use filtration membranes with smaller pore size (0.05 μm), which should catch the smallest particles better [7,24]. In this study the results of the SEMq method were presented as relative numbers of wear particles. Nevertheless, the absolute numbers of wear particles per 1 g of dry tissue were also calculated, being around 1×10^9, which quite agrees with other studies (e.g. [13] and references therein) indicating that the *absolute* values of ED and AP are correct.

5. Conclusion

1. Three different techniques (alkaline, acid, enzymatic) of isolation of PE particles described in literature were tested. All three techniques were modified to obtain pure particles.
2. The modified acid technique was found the best from the point of view of simplicity, reliability, speed and cost of the chemicals.
3. New automated technique for quantification of wear particles, called SEMq, was introduced. It was demonstrated that the technique provides correct, reliable and reproducible results. The SEMq results are very intuitive and clear, which can be employed in verification of other, faster but less transparent techniques, such as LSc [4].
4. The results, obtained by the SEMq technique, are in agreement with our previous findings obtained with LSc [4]. They suggest that the distribution of the numbers of wear particles around TJR is quite non-homogeneous. The numbers of particles in various locations around TJR may differ by as much as one order of magnitude.

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