Examination of trafficking of phagocytosed colloid particles in neutrophils using synchrotron-based X-ray fluorescence microscopy (XFM)

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Abstract Synchrotron-based X-ray fluorescence microscopy (XFM) can localise chemical elements at a subcellular level. 99mTechnetium stannous (TcSn) colloid is taken up by phagocytes via a Complement Receptor 3 mediated phagocytic process. In the current study, XFM was used to examine the intracellular trafficking of TcSn colloid in neutrophils. XFM was performed on TcSn colloid, and neutrophils labelled with TcSn colloid, in whole blood. We developed a set of pixel by pixel analysis and mapping techniques incorporating cluster analysis that allowed us to differentiate neutrophils and artefactual contaminants, and we examined the changes in element distribution that accompany neutrophil phagocytosis of TcSn colloid. Sn became associated with half the neutrophils. Within cells, Sn colocalised with iron (Fe) and sulphur (S), and was negatively associated with calcium (Ca). Despite the high sensitivity of XFM, Tc was not detected. XFM can help clarify the intracellular processes that accompany neutrophil phagocytosis. The subcellular colocalisation of Sn with Fe is consistent with fusion of the colloid-containing phagosome with neutrophil

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granules. The association of Sn with S suggests that proteins rich in S-containing amino acids are present in the phagosome. The negative colocalisation with Ca indicates that ongoing maturation of the TcSn colloid phagosome is no longer calcium dependent one hour after phagocytosis.

Keywords Phagocytosis • Tin colloids • Synchrotron-based X-ray fluorescence microscopy (XFM) • Intracellular trafficking

1 Introduction

Synchrotron-based X-ray fluorescence microscopy (XFM) can determine the subcellular location of specific chemical elements in biological tissues [1, 2]. This technique can detect elements at low concentrations, and has helped clarify the intracellular distribution of trace elements in many cell types, including phagocytic cells [3]. One major advantage that XFM has over related techniques used to study intracellular chemical reactions is that mammalian cells can be studied without chemical fixation and with minimal preparation. No specific dyes are required, and thin layers of cells can be examined without sectioning. This means that the distribution of chemical compounds determined by XFM is more likely to represent their true *in vivo* location, than is the distribution found using other methods which require preparation that is disruptive to cells and their contents. Given these characteristics, XFM has the potential to be utilised to investigate the localisation of pharmaceuticals within human cells.

TcSn colloid (LLK Radpharm Australia) is a commercially available radiopharmaceutical used to label phagocytic leukocytes to image sites of infection [4–8]. When fresh colloid is examined immediately after manufacture it is found to consist of clumps of particles measuring on the order of several micrometers [9, 10]. This compound does not label phagocytic cells at 4°C, at which temperature phagocytosis ceases [4, 5]. Autoradiography of neutrophils labelled with TcSn colloid shows a diffuse pattern of radiation attributable to intracellular radioactive particles, indicating internalisation, which in particles of this size implies phagocytosis, with uptake in 10% of neutrophils [11]. However, phagocytosis is a complex process which can occur via a number of different mechanisms, and can have varying effects on the cell. TcSn colloid uptake by neutrophils is dependent on the I domain of Complement Receptor 3 (CR3) [12], and after TcSn colloid uptake neutrophils are viable, functional and not significantly activated or primed [4, 5, 9, 12]. This resembles type II phagocytosis of opsonised particles which is mediated by CR3 and during which the cell is not activated, and there is no proinflammatory response [13, 14]. In contrast, type I phagocytosis is mediated by immunoglobulin receptors (FcγRs), and results in cell activation and a proinflammatory response. Hence, TcSn colloid cellular uptake has been suggested as a model to study type II phagocytosis [10, 12, 15, 16], although it should be noted that CR3 is also involved in some forms of non-opsonic phagocytosis [17]. Regardless of the mechanism of phagocytosis, the ultimate neutrophil response depends on the nature of the particle internalised, and on the resultant signalling through a complex array of receptors including the CR3 I domain, the CR3 lectin domain (which tends to provoke an inflammatory response [17]), FcγR, the toll-like receptor family, and LPS receptors [13]. Exactly how the neutrophil integrates these signals, the precise differences in the chemical processes which accompany the different subtypes of phagocytosis, and how neutrophil responses differ from those in other phagocytes continues to be a topic of intensive investigation.

In the current study, the subcellular chemical processes associated with the specific subtype of neutrophil phagocytosis that is mediated by the CR3 I domain, and which does not significantly activate the neutrophil, were studied by performing synchrotron-based XFM on neutrophils after TcSn colloid uptake.

2 Materials and methods

2.1 Colloid preparation, blood labelling and neutrophil enrichment

TcSn colloid was freshly prepared from its basic components immediately before use from a commercially available kit (Radpharm Scientific, Australia) using a standard method [8] based on the method of Hanna [4, 5]. 1.5 GBq of freshly eluted sodium [^{99m}Tc]pertechnetate was diluted to 2.5 ml with normal saline in a 5 ml syringe. Five millilitres of 1 mg/ml sodium fluoride in water was added to 640 μg of stannous fluoride as a lyophilised powder in a vacuum-sealed 8 ml glass vial and mixed for 20 s; 0.5 ml of this solution was transferred to the syringe containing the pertechnetate. This syringe was placed on a vertical blood rotator and mixed by slow rotation for one hour.

After rotation 0.1 ml TcSn colloid solution was placed onto a 5-mm square, 1- μm -thick silicon nitride window, and air dried.

Venous blood (20 ml anticoagulated with heparin 5 IU/ml) was taken from a healthy male volunteer. The blood was mixed with 1.5 ml freshly prepared TcSn colloid by continuous rotation on a vertical blood mixer for 1 h at 25°C.

After mixing, neutrophils were enriched. Most RBCs were removed by ice-cold 6% dextran sedimentation. After sedimentation, the supernatant containing leukocytes was pooled and centrifuged (300 g for 12 min at 4°C). Remaining RBCs and platelets were removed by hypotonic lysis and centrifugation (325 g for 6 min at 4°C) repeated twice. Mononuclear cells were separated from the cell pellet by Ficoll-Paque (GE Healthsciences) sedimentation (500 g for 30 min). The enriched neutrophil pellet was resuspended in RPMI-1640 medium.

Using a Shandon Cytospin 4, cells were transferred onto glass slides for light microscopy. The slides were air dried, stained with Diff Quick (Australian Biostain Pty Ltd) and mounted for light microscopy (Fig. 1). The cell suspension (20 μl) was spotted onto 5 mm square 1 μm thick silicon nitride windows to obtain a cell monolayer, then air dried.

Once the cells and TcSn colloid were applied to the windows, 120 h were allowed for radioactive decay before further handling. Light microscopy of the silicon nitride window confirmed a monolayer of cells (Fig. 1).

2.2 X-ray fluorescence microscopy

XFM was performed using the X-ray Fluorescence Microprobe beamline [18] at the Australian Synchrotron in Melbourne, Australia. A 9.9 keV incident X-ray beam energy was selected to cover the L-shell binding energies of Tc ($Z = 43$, L1 edge ~ 3.043 keV) and Sn ($Z = 50$, L1 edge ~ 4.465 keV) as well as biologically interesting first-row transition metals such as Fe and Zn. The beam was focused using a zone-plate with 100 nm outermost zones and a diameter of 160 μm . X-ray fluorescence and scattering originating from the specimen was detected using a single-element Vortex silicon drift diode detector sensitive to photons above 1.1 keV. A single-point measurement was made by illuminating a thin

Fig. 1 Light microscopy of unlabelled neutrophil monolayer after placement on window. *Inset* shows higher power image of representative cells labelled with Diff Quick-95% were neutrophils, with other cells present being predominantly eosinophils

column through the specimen with X-rays, and collecting the X-ray fluorescence emitted by the atoms in this column. Two-dimensional elemental maps were built up by raster-scanning the specimen through the X-ray focus.

The silicon nitride windows were mounted on an *XYZ* sample positioning stage with the plane of the window perpendicular to the incident beam. The detector was positioned near to the 90° scatter peak minimum but rotated upstream by 15° to avoid shadowing from the silicon nitride window. To maximise detection efficiency for fluorescent X-rays, the detector was positioned as close as possible to the sample. The apparatus was operated in a nitrogen-rich environment to reduce the fluorescence from atmospheric argon, whose fluorescence emissions can swamp the signal from nearby trace elements, in particular Tc and Sn.

The TcSn colloid was scanned using the following parameters: scan area 75 $\mu\text{m} \times 100 \mu\text{m}$; beam spot size 0.5 μm ; step size (sampling interval) 1 μm ; dwell time 6 s per pixel. In order to increase the sensitivity for detecting the lower concentrations of Sn expected to be found in the cell sample, the TcSn colloid-labelled neutrophil sample was scanned using similar beam parameters, but with a smaller scan area and a longer dwell time: scan area 50 $\mu\text{m} \times 50 \mu\text{m}$; beam spot size 0.5 μm ; step size (sampling interval) 1 μm ; dwell time 11 s.

The collected spectra were analysed using the MAPS analysis package [19, 20]. Spectral deconvolution was used to resolve the presence of individual chemical elements. The data was interrogated on a pixel-by-pixel basis for the presence of the K-shell X-ray emissions from the elements K, Cl, P, S, Ca, Ni and Zn, and the L-shell X-ray emissions from the heavier elements Tc, Sn and I. The results were displayed as parametric maps.

The individual point spectra obtained during the XFM measurement were fitted to resolve overlay from elemental and other spectral features, and the determined maps of relative elemental content have uncertainties resulting from the measurement and fitting procedures. The measured Sn concentrations were very low, and there is therefore concern that the uncertainty of the measurement and fitting could result in a positive value when one does not actually exist. It is therefore not correct in this situation to interrogate only the fitted quantities: the uncertainties must also be considered. One way to gauge the statistical significance of the measured Sn distribution is to interrogate the fitted value divided by

the one-sigma fitting uncertainty; however, this tends to distort the measured quantities. An alternate approach is to take a mildly pessimistic view of the data, and to consider the fitted values minus the one-sigma fitting uncertainties (thresholded at zero). In view of the fact that we were searching for Sn, and that the default hypothesis was that there was no Sn, we decided to take the second approach to allow for uncertainty in the fitted Sn values by subtracting the one-sigma fitting uncertainty on a pixel-by-pixel basis. When this is done, much of the elemental map goes to zero, indicating that the values in those pixels were consistent with absence of Sn content. The remaining points in the map are likely to represent the actual Sn distribution. For the neutrophil-based sample, any pixel which was found to contain Sn after application of this algorithm was mapped on a pixel-by-pixel basis onto the iodine map to allow a visual assessment of the physical location of Sn. The remaining positive values in the corrected Sn map typically had high significances, i.e., their fitted Sn content was not at all consistent with zero.

2.3 Statistical analysis and parametric mapping

Image manipulation, mathematical calculations and statistical analysis were performed using a combination of Image J 1.43u (NIH, USA), Excel 2010 (Microsoft, USA) and SPSS 19.0.0 (IBM, USA).

For the neutrophil sample, two-step cluster analysis was undertaken of the elemental composition of the cells on a pixel-by-pixel basis (SPSS, log-likelihood distance measure,

Fig. 2 Spectral plot of the TcSn colloid sample. A number of signal peaks consistent with Sn L-shell fluorescence are marked (*). In simple chemical compounds, and when elements are at high concentrations, the energy peaks corresponding to individual elements can be seen on inspection of the spectra. However, for chemically complex samples, such as biological tissues, and when the element confirming that interest is at low concentration, computer-assisted spectral analysis is required

Schwarz's Bayesian Criterion, number of clusters detected automatically). The resultant clusters were then displayed as parametric maps, and overlaid onto the elemental maps to allow visual assessment of the meaning of the clusters.

The cluster analysis results were then used to define those regions of the samples which corresponded to cells (see Section 3). To examine how the concentration of Sn within cells was related to the concentration of other elements, initially correlational analysis (Pearson's correlation coefficient and the nonparametric Spearman's rho) was undertaken on the data within these cellular regions of interest to assess potential relationships. Subsequently, if the correlational analysis did indicate that a relationship existed between Sn and some of the other elements, multiple linear regression was performed on the data from within the cellular regions of interest using Sn as the dependent variable, and the other measured elements as the independent variables (SPSS, stepwise, variables retained in the model if they had a coefficient which was significant at the $p < 0.05$ level and excluded from the model if $p > 0.1$). ANOVA was undertaken to assess the significance of the resultant models against the null hypothesis that all the predictor variables were irrelevant, i.e., to test whether the calculated R squared was significantly greater than zero. The Durbin–Watson statistic was used to confirm the independence of the residuals that resulted from the regression for the final model. t tests were used to assess whether the coefficients (b values) for individual elements calculated in the individual regression equations were indeed significantly greater than zero.

Fig. 3 Parametric maps of elemental location in the TcSn colloid sample after pixel-by-pixel analysis demonstrating the location of chlorine (*Cl*) and tin (*Sn_L*), showing that Sn is present in particles. On light microscopy (not shown), Cl correlates in position with crystals consistent with NaCl

Fig. 4 Parametric maps of ten individual elements for the blood sample. *P* represents the K-shell fluorescence from phosphorous, *Ca* calcium, *S* sulphur, *K* potassium, *Cl* chlorine, *Ni* nickel, *Zn* zinc, *Fe* iron. *I_L* represents the L-shell emissions of iodine and *Sn_L* represents tin. These images show neutrophils as well as a number of other smaller foci of high elemental concentration which represent contaminants

3 Results

The TcSn colloid sample produced a robust Sn signal (Fig. 2). The spatial distribution of Sn corresponded to multiple small particles (Fig. 3). The characteristic fluorescent X-rays of Tc were not detected.

Fig. 5 *Sn: Fit-sigma* Parametric map showing (marked as yellow) those pixels in the blood sample in which Sn concentration as determined by the fitting algorithm survived the one sigma uncertainty correction (see text). *I* Elemental iodine map for this sample. *Sn+I* Superimposition of Sn signal onto iodine map shows the distribution of Sn is consistent with cellular association of Sn-containing particles

Fig. 6 Parametric map of the cluster number of individual pixels used to define the location of cells, and to exclude contaminants. In the second image, *white* indicates pixels with membership in cell-based clusters (cluster numbers 1, 2 and 6) and *black* indicates pixels with membership in non-cell clusters (clusters 3, 4 and 5). Corresponding iodine map allows visualisation of the physical location of these clusters

The TcSn colloid-labelled cells produced strong signals for elements known to have high cellular concentrations (K, P), weaker signals for elements known to have lower intracellular concentrations (Fe, Zn), a relatively strong signal for S and a very weak signal for Sn (Fig. 4). Unexpectedly, one of the highest signals obtained from the cells was compatible with L fluorescence of iodine, indicating a high level of cellular I. This observation proved serendipitous, assisting in visualising the location of individual cells.

The parametric map of the Sn values after the one-sigma correction and the resultant projection onto the iodine cellular map is shown in Fig. 5. Based on assessment of these parametric images, Sn was present in 22/42 (52%) of neutrophils. The Sn content varied between cells. No significant Tc signal was detected.

With cluster analysis, six distinct clusters were obtained (Fig. 6). Cluster 5 corresponded to empty space on the slide, while cluster 3 represented focal regions with very high Cl concentration consistent with NaCl crystals, and cluster 4 corresponded to a relatively small number of pixels with high sulphur levels, high potassium levels and high calcium levels which most likely correspond to contaminant dust particles containing the relatively common environmental compounds potassium sulphates and/or calcium phosphates or to small fibrin clumps. The other three clusters corresponded to the physical location of cells,

Table 1 Correlation between the local concentration of Sn and that of the other elements was investigated as a first pass by simple correlational analysis

		Sn	K	S	Fe	Ca	P	Zn	Cl	Ni
Sn	Pearson correlation	1.0	0.103 ^a	0.275 ^a	0.309 ^a	−0.018	0.044	0.006	0.074 ^a	0.066 ^a
	Sig. (2-tailed)		0.000	0.000	0.000	0.432	0.053	0.781	0.001	0.004
	<i>N</i>	1961	1961	1961	1961	1961	1961	1961	1961	1961
Sn	Spearman's rho	1.0	0.087 ^a	0.212 ^a	0.193 ^a	0.003	0.031	0.024	0.011	0.049
	Sig. (2-tailed)		0.000	0.000	0.000	0.891	0.167	0.288	0.630	0.029
	<i>N</i>	1961	1961	1961	1961	1961	1961	1961	1961	1961

Suggestive correlations were found using parametric testing (Pearson's correlation coefficient) and were still seen for some elements using nonparametric testing (Spearman's rho) confirming the relationship is unlikely to be due to a small number of possible outliers

^aCorrelation is significant at the 0.01 level (two-tailed)

Table 2 Summary of the results of the stepwise multiple linear regression performed on intracellular regions with Sn as the dependent variable and the other measured elements as the independent variables (see text for details)

Model	<i>R</i>	<i>R</i> Squared	Adjusted <i>R</i> Squared	<i>b</i>	Standard error of <i>b</i>	Standardised coefficients (Beta)	<i>t</i> ^a	Significance ^a
1	(Constant)	0.309	0.095 ^b	(-0.078)	0.028		-2.799	0.005
	Fe			0.218	0.015	0.309	14.369	<0.001
2	(Constant)	0.330	0.108 ^c	(-0.248)	0.042		-5.922	<0.001
	Fe			0.158	0.019	0.225	8.508	<0.001
	S			0.018	0.003	0.143	5.400	<0.001
3	(Constant)	0.339	0.115 ^d	(-0.198)	0.044		-4.499	<0.001
	Fe			0.162	0.019	0.229	8.703	<0.001
	S			0.020	0.003	0.154	5.808	<0.001
	Ca			-0.004	0.001	-0.079	-3.634	<0.001

^aTwo-tailed t-test for assessment of significance of individual elements in the model^b $F = 206$; $p < 0.001$ ^c $F = 119$; $p < 0.001$ ^d $F = 84$; $p < 0.001$

as can be seen from Fig. 6. These clusters were used to define ROIs—only pixels within the “cellular” clusters 1, 2 and 6 were subjected to further analysis by multiple linear regression to examine intracellular elemental concentrations.

With regards to subcellular trafficking of Sn, the correlation analysis did suggest a relationship between Sn levels and the levels of some of the other elements studied (Table 1). Stepwise multiple linear regression was therefore proceeded with. This suggested three possible models for the spatial relationship between Sn and the other elements (Table 2), one in which Sn was colocalised with Fe, one in which it was colocalised with Fe and S, and the “best” model in which there was a significant positive relationship between the local concentration of Sn and that of Fe and S, and a small negative association with Ca ($F = 84$, $p < 0.001$, Durbin–Watson statistic 1.79, which is relatively close to 2 and consistent with independent errors). Further details on the multiple linear regression are included in the [electronic supplementary material](#). This model gives an adjusted R^2 of 0.11, indicating that it accounts for 11% of the variation in Sn concentration. Once the effects of these three elements (Fe, S and Ca) were accounted for in the model, the other chemical elements that had been measured were not significantly colocalised with Sn (see [electronic supplementary material](#)).

4 Discussion

This study demonstrates that synchrotron-based XFM can be used to examine the chemical processes which accompany the CR3-mediated phagocytosis of TcSn colloid particles by neutrophils. After phagocytosis, the Sn within the labelled neutrophils was found to be spatially associated with Fe and S. Neutrophil granules contain Fe, much of which is in the haem molecules of the gp91^{PHOX} protein, which is a key component of the NADPH oxidase system that drives the respiratory burst, and which is found in specific and tertiary granules, but not in azurophilic granules [21]. Azurophilic granules do contain iron in the haem molecules of myeloperoxidase, which is not present in the other granules. However, specific granules contain far more Fe than do azurophilic granules [22]. Specific granules are the main source of phagosomal gp91^{PHOX} [23], while tertiary granules deliver this compound to the cell surface [23]. This suggests that local Fe concentration within the phagosome is determined by fusion with specific granules rather than azurophilic granules. Hence, the most likely explanation for the subcellular association of Sn with Fe found in our study is that the TcSn colloid-containing phagosomes fuse with neutrophil granules, and more specifically the gp91^{PHOX}-containing specific granules, within the first hour after phagocytosis. However, previous studies have shown that there is no evidence of neutrophils undergoing the respiratory burst as a result of TcSn colloid labelling [4, 5, 9, 12]. Hence, despite the colocalisation of at least one of the components of the NADPH oxidase to the TcSn colloid-containing phagosomes, this system is not activated.

Within neutrophils much of the sulphur is present in proteins as a component of specific amino acids (e.g. methionine and cysteine). The colocalisation of Sn with S therefore most likely indicates that proteins rich in the S-containing amino acids are recruited to the phagosome. This association with sulphur is less likely to be attributable to an association of S with Fe in so-called iron-sulphur compounds, because these are the product of mitochondria, and neutrophil mitochondria play little role in neutrophil metabolism except during apoptosis [24]. Few iron-sulphur compounds are found in neutrophil granules [25]. In addition, the multiple linear regression result implies that the association of S with Sn

is not entirely due to that with Fe, and indicates that other S-rich compounds are recruited. Another sulphur compound which has been shown to be involved in neutrophil function and could be involved in this instance is the gaseous transmitter hydrogen sulphide, which prevents neutrophil apoptosis [26] (as does CR3-mediated phagocytosis).

The negative association with calcium was more surprising, given that it has been reported that neutrophil phagocytosis is Ca dependent and active phagosomes are associated with local increases in Ca concentration [27]. However, more recently, it has been shown that although the initial phagocytic event in neutrophils is calcium dependent, when phagocytosis is CR3 mediated the resultant increase in local Ca concentration lasts for only 2–3 min [28]. Later recruitment of azurophilic granules to the phagosome is calcium independent [29]. This temporal difference in Ca response could explain why there was no positive association of Ca with Sn, given that 1 h was allowed for TcSn colloid uptake by neutrophils. It is also possible that 1 h after internalisation of an inorganic particle such as TcSn colloid, which is phagocytosed via an I domain-related CR3 process [12] and which has no pathogen-associated molecular patterns to stimulate other neutrophil receptors, that the phagosome has reached a quiescent stage, and represents a metabolically inactive region of the cell, with Ca levels therefore being higher in other metabolically active subcellular compartments.

This study confirms previous findings about the composition of TcSn colloid. These particles contain Sn, but not significant amounts of Cl, as previously described using SEM and energy-dispersive X-ray analysis (EDAX) [9]. McClelland also reported that, in addition, the particles contain O but not F [9], suggesting they consist primarily of stannous oxide. The majority of the Cl found in the TcSn colloid solution residue after dehydration is in separate NaCl salt crystals [9]. Although XFM is capable of detecting very low concentrations of elements, in the current study we were not able to directly identify the presence of Tc in TcSn colloid. This is probably at least in part because of the very small quantity of Tc that is present in TcSn colloid. Specific measurements of the relative amounts of Tc and Sn in TcSn colloid have not previously been reported, and such measurements should be formally undertaken. However, an idea of the relative amounts of these elements present can be obtained by assuming that the Tc eluate used in manufacture is very fresh (and hence consists of 99mTc, with negligible other isotopes of Tc present). Based on this assumption, there are approximately 4.7×10^{13} atoms of Tc in 1.5 GBq of Tc99m. This compares to 2.5×10^{18} atoms of Sn in 640 µg of SnF₂, with 10% of this amount of Sn ultimately mixed with the Tc. If we further assume that all available Sn and Tc is incorporated into colloid, and that Tc is relatively evenly dispersed in the colloid, the TcSn colloid sample should contain approximately 5,000 Sn molecules for every Tc molecule. Filtration experiments have confirmed that in TcSn colloid Tc is physically associated with these Sn-containing particles [4, 5, 9], though it is worth noting that previous studies using EDAX did not confirm the presence of Tc in TcSn colloid. In addition, the K emissions of chlorine overlie the energy range of the L emissions of Tc, and as Cl was present these Cl emissions could be obscuring low-level emissions from Tc.

As we did not detect Tc in TcSn colloid, it is not surprising that we did not detect a Tc signal from the neutrophils labelled with TcSn colloid, a sample in which Tc was at an even lower relative concentration and which consists of an even more complex mixture of elements. Other investigators using the related technique of electron probe microanalysis have reported that Tc can be detected at the subcellular level within mitochondria in animal cells, but only after cultured chick embryonic heart cells were directly bathed in Tc-MIBI at concentrations of Tc much higher than is achieved at the cellular level in clinical

nuclear medicine practice, and then thinly sectioned [30]. With ongoing evolution of XFM techniques, including the development of tomographic analysis [31], we hope to eventually be able to confidently identify the location of intracellular Tc in samples subjected to minimal preparation.

From the point of view of understanding the labelling process of phagocytic cells with TcSn colloid, even though the current study did not detect Tc, it is still likely we have visualised the location of TcSn colloid in relation to the neutrophils by examining the distribution of Sn. As discussed above, when TcSn colloid is manufactured using ^{99m}Tc , and the particles are then filtered out of suspension, these particles are radioactive, and so must contain Tc [4, 5, 9]. Hence, by mapping the location of Sn within neutrophils we can infer the location of Tc, that is, with XFM we can use Sn as a “tracer” for the location of Tc. In the clinical setting TcSn colloid has consistently proven efficacious in detecting focal infection [4–8]. Some studies have reported pessimistically on TcSn colloid leukocyte labelling efficiency [9]; however, such studies may not use the standard labelling method. For example, citrate anticoagulation is sometimes used instead of heparin [9]. Heparin increases neutrophil CR3 expression in a dose-dependent fashion [31], which would be expected to increase TcSn colloid uptake, while citrate induces a relatively suppressed CR3 response [32, 33]. The current study indicates that after cell labelling was undertaken in heparinised whole blood, and the neutrophils were then separated from other blood components, about half of the separated neutrophils contained TcSn colloid, while half did not contain measurable amounts of Sn. Given these results, and given that TcSn colloid neutrophil uptake is a CR3-mediated process [12], citrate anticoagulation should be avoided, whether using this pharmaceutical for investigating phagocytic function or for imaging purposes.

Iodine is present in normal neutrophils at relatively low concentrations [34] which would not explain the high level of signal intensity attributable to iodine in this study. The neutrophil enrichment process in the current study utilised Ficoll-Paque, which contains the iodine-rich compound diatrizoate. The high iodine concentration observed is probably due to diatrizoate binding to the cell surface, or being taken up by the cells. This finding is of additional interest in medical imaging because diatrizoate is a component of some radiological contrast media.

5 Conclusion

The current study demonstrates that synchrotron-based XFM can confirm the association of Sn-based colloid particles with individual human neutrophils, and can be used to investigate the cellular handling of Sn-containing phagosomes created by CR3-mediated phagocytosis. When TcSn colloid is mixed with whole blood, Sn-based colloid particles became associated with half of the neutrophils. After the TcSn colloid is taken up by neutrophils there is a subcellular colocalisation of Sn with Fe, suggesting that Sn is present within phagosomes that have fused with specific granules. It is most likely this Fe is within the protein gp91^{PHOX}, suggesting colocalisation of Sn with components of the NADPH oxidase system, a key component of the respiratory burst, although the respiratory burst is not activated by neutrophil TcSn colloid labelling. The association of neutrophil Sn with S suggests that proteins rich in the S-containing amino acids (such as methionine and cysteine) are associated with the phagosome. There is a negative association of Sn with Ca location, which indicates that at one hour after phagocytosis of these particles the process is no longer

stimulating a local Ca influx. This implies either that ongoing maturation of the neutrophil phagosome is not calcium dependent or that the process is completed, and the cell has returned to a “resting” state.

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