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Highly stable monodisperse PEGylated iron oxide nanoparticle aqueous suspensions: a nontoxic tracer for homogeneous magnetic bioassays[†]

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Uniformly sized and shaped iron oxide nanoparticles with a mean size of 25 nm were synthesized via decomposition of iron-oleate. High resolution transmission electron microscopy and Mössbauer spectroscopy investigations revealed that the particles are spheres primarily composed of Fe₃O₄ with a small fraction of FeO. From Mössbauer and static magnetization measurements, it was deduced that the particles are superparamagnetic at room temperature. The hydrophobic particles were successfully transferred into water via PEGylation using nitrodopamine as an anchoring group. IR spectroscopy and thermogravimetric analysis showed the success and efficiency of the phase transfer reaction. After the PEGylation, the particles retained monodisperse and their magnetic core remained intact as proven by photon cross-correlation spectroscopy, ac susceptibility, and transmission electron microscopy. The particle aqueous suspensions revealed an excellent water stability over a month of monitoring and also against temperature up to 40°C. The particles exhibited a moderate cytotoxic effect on *in vitro* cultured bone marrow-derived macrophages and no release of inflammatory or anti-inflammatory cytokines. The PEGylated particles were functionalized with Herceptin antibodies via a conjugation chemistry, their response to a rotating magnetic field was studied using a fluxgate-based setup and was compared with the one recorded for hydrophobic and PEGylated particles. The particle phase lag rose after labeling with Herceptin, indicating the successful conjugation of Herceptin antibodies to the particles.

1 Introduction

Iron oxide nanoparticles (NPs) have become an indispensable candidate for a broad range of nanoparticle-based diagnostics and therapeutics owing to their excellent physiochemical properties. Manipulation of magnetic NPs in dynamic magnetic fields (e.g. alternating and rotating) has recently become a potent tool for detection of proteins and molecules bound to

the NPs.^{1–6} To conduct a successful homogeneous magnetic bioassay, particularly in a rotating magnetic field (RMF), the iron oxide NP aqueous suspensions relaxing via the Brownian mechanism ($d_{\text{core}} > 24$ nm) are required.

Fabrication of iron oxide biomarkers starts from the dimensionally and magnetically controlled synthesis of particles and characterization of their structural and functional properties. In the last two decades, there has been a growing trend to employ high temperature decomposition of various iron precursors, such as iron acetylacetonate Fe(acac)₃,⁷ iron-oleate,⁸ iron pentacarbonyl Fe(CO)₅,⁹ and hydrated iron(III) oxide FeO(OH)¹⁰ to fabricate iron oxide NPs. By having a precise control over the synthesis parameters, these methods offer crystallized monodisperse particles with appropriate magnetic properties but, solely dispersible in non-polar solvents. The next challenge is the transfer of hydrophobic NPs into water, an essential criterion for biological applications. Further topics are the particle stability and cytotoxicity. These issues influence directly the particle's life time in the circulatory system as well as their biodistribution. It appears that the particle surface chemistry has to be meticulously engineered.

Among different organic compounds investigated hitherto

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for surface modification of iron oxide NPs, poly(ethylene glycol) (PEG) has gained a lot of attention due to its promising physiochemical properties. To conjugate PEG to particles, different anchoring groups such as silane,^{11,12} dopamine,¹³ phosphate,¹⁴ nitrodopamine,¹⁵ and multidentate catechol have been already utilized.¹⁶ Xie et al. transferred and stabilized oleic acid/oleylamine coated NPs by PEG-dopamine and demonstrated a remarkable reduction in non-specific uptake by macrophage cells.¹⁷ Amstad et al. showed higher stability of the PEG compounds bound to iron oxide NPs by nitrocatechols than catechols.¹⁵ Having anchored PEG to ultrasmall iron oxide nanoparticles by phosphate groups, Tromsdorf et al. fabricated an efficient T_1 MR contrast agent and reported a strong dependency of the particle uptake by macrophages on the PEG packing density.¹⁴ Recently, Liu et al. synthesized iron oxide NPs covered with a highly dense PEG layer via inducing plenty of carboxylate binding sites using poly(acrylic acid) as the exchanging ligand and showed their high performance in *in vivo* cancer tumor imaging.¹⁸ To date, a handful of studies has shown the capability of PEG for stabilization of small iron oxide NPs ($d_{\text{core}} \approx 10$ nm).^{13–15,17,18} Nonetheless, the feasibility of transferring larger iron oxide NPs ($d_{\text{core}} \approx 25$ nm) with PEG and their further functionalization for biomedical implications have not yet been investigated.

In this study, oleic acid capped iron oxide NPs with a mean core size of 25 nm were fabricated via a procedure published elsewhere.¹⁹ Their crystal structure and phase composition were thoroughly studied by high resolution transmission electron microscopy (HRTEM), selected area electron diffraction (SAED) and Mössbauer spectroscopy. The field and temperature dependent magnetization measurements were performed with a Quantum Design Magnetic Property Measurement System (SQUID-MPMS). The nitrodopamine and hydroxyl activated PEG-nitrodopamine (HO-PEG-nitrodopamine) were synthesized, and characterized by ^1H NMR and IR Spectroscopy. These particles were transferred into water via a mild ligand exchange process. The success and efficiency of the employed phase transfer strategy were assessed by IR, thermogravimetric analysis (TGA) and ac susceptibility (ACS). The stability of particle aqueous suspensions against storage time and temperature has been rigorously evaluated by photon cross-correlation spectroscopy (PCCS). The particle cytotoxicity was explored by conducting a series of cytokine and lactate dehydrogenase (LDH) assays on bone marrow-derived macrophage cells. The particle uptake by macrophages was probed with transmission electron microscopy and electron energy-loss spectroscopy (EELS). Next, the particles were functionalized with Herceptin antibodies via a conjugation reaction. The change in the particle dynamic response after the PEGylation and labeling with Herceptin was investigated using a fluxgate-based RMF setup.

2 Results and discussion

2.1 Characterization of hydrophobic oleic acid capped NPs

The oleic acid capped NPs (denoted by NP-OA) were characterized from different aspects as described in the Supporting Information†. Firstly, the magnetic core properties have been examined due to their huge impact on particle biomedical application. In our previous publication,¹⁹ we have shown that delayed nucleation and quickly terminating growth are the plausible formation mechanisms of large monodisperse NPs in this specific synthesis reaction. An overall TEM image of the NP-OA is shown in Fig. 1(a). As can be clearly seen, the particles have uniform shape and size distributions. The particles reveal a lognormal size distribution with a mean diameter of 25 nm ($\sigma_c = 0.05$) as plotted in Fig. 1(b). The selected area electron diffraction (SAED) pattern taken from accumulated layers of particles is displayed in Fig. 1(c). The diffraction rings corresponding to both Fe_3O_4 and FeO phases can be identified as annotated in the figure. Apparently, the rings which can be attributed to both Fe_3O_4 and FeO interplanar spacing are more prominent (e.g. $\{400\}_{\text{Fe}_3\text{O}_4}$ and $\{200\}_{\text{FeO}}$). Conversely, the rings solely connected to Fe_3O_4 (e.g. $\{220\}$ and $\{511\}$) are less pronounced. It can be concluded that the particles are composed of both phases. To examine the particle crystallinity, the HRTEM technique was exploited. The image taken from a single particle is depicted in Fig. 1(d). The crystalline planes oriented parallel to the electron beam with $d_{\text{spacing}} \approx 0.2$ nm can be ascribed to $\{400\}_{\text{Fe}_3\text{O}_4}$ and also tentatively to $\{200\}_{\text{FeO}}$ ($d_{\text{spacing}} \approx 0.215$ nm). Generally, the particles are well ordered at the outer layers and less crystallized in the core. This phenomenon can be presumably related to the existence of a pure Fe_3O_4 phase at the shell and a mixture of both phases at the core. Admittedly, a detailed phase analysis can not be successfully accomplished by electron diffraction and HRTEM techniques since Fe_3O_4 and FeO share an identical oxygen sublattice.

Therefore, to do a quantitative phase analysis, Mössbauer (MS) absorption spectroscopy was employed. MS measurements were carried out at different temperatures, 15 K, 215 K and 295 K (RT), to gain a full insight into the different magnetic states of the particles. At 15 K, the magnetic moment fluctuations of the NPs with a diameter of a few tens of nanometer are expected to be much slower than nuclear Larmor precession ($\approx$ GHz). Therefore, relaxational line broadening is negligible. As a general trend, the magnetic hyperfine pattern consists of sharp resonance lines at low temperature which broaden and the splitting collapses as temperature increases due to the increase of the fluctuation rate of the magnetic moments of the NPs. At 215 K, right above the Néel temperature T_N of FeO, we expect to observe a nonmagnetic

Fig. 1 (a) Typical TEM micrograph of NP-OA nanoparticles, (b) their size histogram, (c) SAED pattern taken from accumulated layers of particles, and (d) HRTEM micrograph of a single 25 nm NP-OA.

contribution corresponding to FeO which is easily distinguishable from Fe_3O_4 . A further RT spectrum was added for completeness.

The recorded MS spectra are displayed in Fig. 2. The low temperature spectrum was fitted using five subspectra with the hyperfine parameters corresponding to magnetite²⁰ and two hyperfine field distributions. The analyzed hyperfine parameters are summarized in Table 1. In the 15 K spectrum, there is a broad structure lying below the six magnetic lines which is not expected for pure magnetite. This broad feature can be attributed to FeO and an interfacial FeO/ Fe_3O_4 phase. Owing to the complexity of FeO's low temperature spectrum,²¹ no detailed analysis has been carried out on this spectral component. For an approximate description, we used two hyperfine field distributions with isomer shifts of ≈ 1.14 mm/s (D1) and ≈ 0.22 mm/s (D2), being tentatively attributed to FeO and the aforementioned interfacial phase, respectively. Their spectral fractions are 18% and 20%.

For the spectra taken at 215 K and RT, we applied a different fitting strategy using two subspectra corresponding to magnetite A- and B-site, a hyperfine field distribution simulating the relaxation effects, and a non-magnetic contribution of the fast fluctuating moments of the superparamagnetic particles with a isomer shift (IS) ≈ 0.46 mm/s. This fitting strategy is similar to the one presented by Dézsi et al.²⁰ for magnetite nanoparticles above the Verwey transition. It is worth noting that the ad hoc introduced hyperfine field distribution provides no physically adequate description of eventual dynamic line broadening in this temperature range. Nevertheless,

Table 1 Analyzed hyperfine parameters of ^{57}Fe Mössbauer spectrum of 25 nm nanoparticles measured at 15 K

Component	IS^a (mm/s)	Q^b (mm/s)	B_{hf}^c (T)	A^d (%)	w^e (mm/s)
a	0.25	0	50.5	37	0.2
b1	0.39	0	52.7	30	0.23
b2	0.57	0.01	51.0	6.6	0.47
b3	0.85	-0.55	48.9	8	0.30
b4	0.73	1.92	37.0	4.6	0.15
b5	0.70	0.01	48.8	14.4	0.37

^a isomer shift relative to Fe metal at RT; ^b quadrupole splitting; ^c hyperfine magnetic field at ^{57}Fe nuclei; ^d area corresponding to each spectral component; ^e Line width.

Fig. 2 Mössbauer spectra of NP-OA particles measured at different temperatures. The red lines are least-squares fits to a superposition of different spectral components.

it gives sufficient information for an estimate of the hyperfine parameters. Another feature of these spectra is the appearance of a paramagnetic component with a IS ≈ 1.51 mm/s, characteristic of Fe^{2+} in FeO.²¹ The spectral fraction of this component matches well with the fraction obtained for FeO at low temperature measurements. Note that at room tempera-

ture the spectrum collapses almost completely, implying that the major fraction of the particle moments is fluctuated faster than the nuclear Larmor precession frequency.

To identify whether these large NPs are superparamagnetic, field and temperature dependent magnetization measurements were performed on the immobile samples (see the Supporting Information†). The $M-H$ curve showed the presence of a small coercivity field ($\mu_0 H_c = 0.65$ mT) which indicates that the particles are superparamagnetic at RT. Field-cooled (FC) and zero-field-cooled (ZFC) magnetizations are plotted in Fig. 3. In the ZFC, the magnetic moment rises slightly up to ≈ 180 K where it soars abruptly and peaks at 260 K. The temperature whereby the sudden increase appears coincides with T_N of FeO. Switching from antiferromagnetic to paramagnetic spin configuration in FeO at T_N can be accounted for this observation. Notably, the superparamagnetic blocking temperature T_B , a temperature at which highest magnetization is reached, is 260 K well below RT. Note that the blocking temperatures estimated from magnetic and Mössbauer measurements agree well.

Fig. 3 FC and ZFC magnetizations as a function of temperature varying from 5 to 295 K. The measurements were performed on the immobile NP-OA particles.

2.2 PEGylated NPs: synthesis and characterization

To link PEG moieties to the particles, nitrodopamine with high binding affinity to the interfacial iron atoms was chosen as an anchoring group. The synthesis procedure of both nitrodopamine and the conjugated HO-PEG-nitrodopamine are described in detail in the Supporting Information†. The success of the reactions were proven by ^1H NMR and IR spectroscopy. The NMR spectra (Fig. S1†) and their interpretation can be found in the Supporting Information†. Fig. 4 depicts the IR spectrum of HO-PEG-nitrodopamine. A band

appears at 1530 cm^{-1} can be attributed to NH deformation vibration in secondary amides.²² Another absorption band seen at 1665 cm^{-1} can be related to the C=O stretching vibration of secondary amides. Besides, the absorption band at 1720 cm^{-1} may be an indication of a slight remaining trace of unreacted NHS-PEG. Additionally, the absorption bands appear at $1050\text{--}1200\text{ cm}^{-1}$ are ascribed to C-O-C stretching vibration of PEG chains.^{18,22} After knowing the chemical structure of the synthesized hydrophilic ligands, we transferred the NP-OA into water via a simple room temperature process by exchanging their original OA molecules with HO-PEG-nitrodopamine counterparts as schematically illustrated in Fig. 5. The procedure is explained in the Supporting Information†. In the following, the hydrophilic PEGylated NPs are designated as NP-PEG.

Fig. 4 IR spectra of NP-OA, HO-PEG-nitrodopamine and NP-PEG.

The success of the ligand exchange process were examined by IR. The IR spectra of both NP-OA and NP-PEG samples are displayed in Fig. 4. The IR spectrum of the NP-OA reveals the characteristic band of Fe-O bond at 580 cm^{-1} , C=O ($1440\text{--}1600\text{ cm}^{-1}$) and C-H stretching $2800\text{--}3000\text{ cm}^{-1}$ groups. As can be seen in the spectrum of the NP-PEG, the absorption bands assigned to HO-PEG-nitrodopamine remain unchanged after being exchanged with OA molecules. Furthermore, the band attributed to the Fe-O bond (i.e. 580 cm^{-1}) retains as it was in the NP-OA particles.

The amounts of the capping molecules prior and after the ligand exchange were assessed by TGA. The weight loss of NP-OA and NP-PEG particles as a function of temperature are plotted in Fig. 6. The total weight of OA molecules pyrolysed between 25°C and 600°C is $\approx 26\%$, happening in two distinct steps. The first weight loss occurs between 180°C and 270°C which is followed by the second step terminating at 390°C .

Fig. 5 Schematic depiction of the phase transfer strategy via exchanging OA molecules with hydrophilic HO-PEG-nitrodopamine groups; Typical TEM micrograph of hydrophilic NP-PEG particles is shown.

The amount of the OA molecules per gram of particles was calculated to be ≈ 1.2 mmole. It appears that OA molecules are pyrolysed in two temperature regimes. For further investigation, differential scanning calorimetry (DSC) analysis was performed on this sample and the results are plotted in the inset of Fig. 6. The presence of two distinct derivative endothermic peaks can be easily discerned, implying that the bound OA molecules have two different binding strengths.

Fig. 6 TGA results obtained for NP-OA (—) and NP-PEG stabilized with 28 (Δ) and 9 ($\bullet$) $\text{mg}_{\text{PEG}}/\text{mg}_{\text{NPs}}$; the inset is the DSC results of NP-OA.

After the PEGylation, the total weight loss percentage increases substantially. The ligand exchange reaction was performed at two PEG:NPs mass ratios to study the impact of

available PEG molecules on their packing density. The total weight loss of PEG groups on the NP-PEG particles stabilized with 28 (Δ) and 9 ($\bullet$) $\text{mg}_{\text{PEG}}/\text{mg}_{\text{NPs}}$ are 92% to 66% and 88% to 62%, respectively. The bigger and smaller values were respectively obtained by assuming that either all OA molecules have been completely removed or have been fully remained on the particle after the PEGylation. Since the temperature ranges in which OA and PEG molecules are pyrolysed are unresolved, only a weight loss range can be given. Interestingly, the weight percentage of the capped PEG groups is virtually independent of the PEG:NPs mass ratio chosen for the exchange reaction. This indicates that solely a single PEG layer encapsulates the particles regardless of the amount of PEG groups added to the reaction. The weight loss percentages measured between 25 °C and 600 °C, for the scenario that all OA molecules remained bound to the particles, were converted into the number of PEG per particle surface area provided that the particles are spherical with density of 5.18 g cm^{-3} . The number of PEG per square nanometer of the particle surface was estimated to be $1\text{--}1.5 \text{ PEG nm}^{-2}$ for both mass ratios. This finding is in a good agreement with the packing density previously approximated for PEG derivative dispersants.^{15,18}

The hydrodynamic size number distribution of NP-OA and NP-PEG was measured by PCCS. The NP-OA particles reveal a narrow size distribution with a mean size of $30 \pm 3.5 \text{ nm}$. No trace of clustering was detected. Knowing that the mean core size is 25 nm and the length of an OA molecule is 2–2.5 nm,¹⁷ it can be assumed that the particles are covered with a mono layer of OA molecules. After the PEGylation and suspending in water, the particle hydrodynamic size grew

Fig. 7 Number weighted hydrodynamic sizes of NP-PEG particles measured as a function of measurement temperature and storage time.

to 60 ± 8 nm. This observation coincides well with respectively ≈ 30 nm and ≈ 40 nm enlargement in hydrodynamic size after conjugating PEG moieties with a molecular weight of 5 and 3 kDa reported previously.^{15,17,18} The stability of the NP-PEG particles in water and PBS (100 mM) as a function of storage period and temperature was monitored by PCCS and the results are plotted in Fig. 7. The measurement conditions are provided in the Supporting Information†. As can be seen, no remarkable change in the hydrodynamic size was observed after a month of storage in water. Besides the particle suspensions in PBS reveal an excellent stability in such a highly ionic medium and their hydrodynamic size remains unchanged within one week of monitoring. Moreover, the particle suspensions show an exceptional stability against temperature up to 40°C.

The impact of the PEGylation on the particle static and dynamic magnetic properties was investigated by SQUID-MPMS and ACS. The recorded $M-H$ curves are plotted in Fig. 8(a). The saturation magnetizations M_s values are 240 and 234 kA m⁻¹ for NP-OA and NP-PEG samples, respectively. The findings indicate that the PEGylation has an insignificant influence on the particle static magnetization. The particle response to dynamic magnetic fields changes remarkably after the PEGylation as examined by ACS. ACS measurements were performed on particle suspensions at a magnetic field amplitude of 95 μT from 1 kHz to 0.6 MHz. The recorded real and imaginary parts are displayed in Fig. 8(b). Note that in ACS both Néel and Brownian relaxation mechanisms contribute and the quickest one dominates. The Brownian peaks of NP-OA and NP-PEG particles are at 20 kHz and 5 kHz, respectively. The observed shift towards lower frequencies after the PEGylation agrees with 30 nm enlargement in the particle hydrodynamic diameter measured by PCCS.

Fig. 8 (a) $M-H$ magnetization curves and (b) real and imaginary part of the frequency dependent ac susceptibility of NP-OA and NP-PEG particle suspensions taken at 295 K. The iron concentration of ACS samples was set to 10 mM Fe.

The Néel-relaxation-dominated particles produce a constant real part signal for frequencies smaller than their characteristic relaxation frequencies and thus the drop seen in the real part is solely related to the Brownian-relaxation-dominated particles. Accordingly, we can estimate the Néel particles' fraction k introduced by Yoshida et al.²³ by calculating the percentage that the real part signal drops within the measurement frequency range. The fraction of the Néel particles was found to be $k=6.6\%$ for both NP-OA and NP-PEG samples.

2.3 Cytotoxicity assessment

Macrophages are one of the first cell lines encounter invading microorganisms and are important mediators of inflammation. In this study, bone marrow-derived macrophages were treated with the NP-PEG suspensions at three iron doses, 0.3 (labeled by 1:1000 (NP-PEG:DMEM)), 3 and 30 μg_{Fe} mL⁻¹ to study their impact on the cytokine production of macrophages. The chemicals and experimental procedures used in the assays

are provided in the Supporting Information†. The levels of IL-6 and IL-10 as inflammatory and anti-inflammatory markers were determined by ELISA and the results are plotted in Figs. 9(a) and (b). For comparison, the cytokine induced in cells treated with LPS, a strong inflammatory component of Gram-negative bacteria, is plotted too. No release of inflammatory or anti-inflammatory cytokines was observed even at the highest tested particle concentration which exceeds a typical iron concentration used in nanoparticle-based MRI experiments (i.e. 1–20 mg kg⁻¹).¹²

Fig. 9 Amounts of the IL-6 and IL-10 cytokines released by macrophages after being incubated with NP-PEG suspensions for 24 h determined by ELISA. n.d. = not detected

Next, we evaluated the particle cytotoxic effect on *in vitro* cultured bone marrow-derived macrophages. The cultured macrophages were treated with the NP-PEG suspensions at iron doses mentioned earlier. Generally, the release of the cytoplasmic enzyme lactate dehydrogenase (LDH) into the culture medium is an indication of plasma membrane disruption. Hence the levels of cell cytotoxicity were determined by the release of LDH into the culture supernatants as plotted in Fig. 9(c). The particles reveal a moderate cytotoxic effect on macrophages after 24 h of incubation.

2.4 Particle uptake by macrophages: ultrastructural analysis

To examine the particle uptake by macrophages, we have performed a series of TEM experiments on in-situ ultrathin sections of the treated macrophages. The sample preparation and operation conditions are provided in the Supporting Informa-

tion†. Fig. 10(a) shows a single cell treated with the NP suspensions at an iron concentration of 30 $\mu\text{g}_{\text{Fe}} \text{mL}^{-1}$. The intracellular presence of the NPs taken up by the cell is clearly observed. Apparently, both single particles (indicated by arrows) and micro size clusters (captured in circles) were engulfed by the cell via typical phagocytoses and accommodated in primary lysosomes. The area captured in a square is magnified and displayed in Fig. S3(a)†. A survey view of the cell treated with the particles at an iron concentration of 0.3 $\mu\text{g}_{\text{Fe}} \text{mL}^{-1}$ is depicted in Fig. 10(b). It can be easily seen that the particle uptake reduces considerably as the particle concentration decreases. Interestingly, in this concentration, the engulfed particles are mainly individual NPs (black arrows) as can obviously be seen in Fig. S3(b)†. However, a few nanoscale particle clusters have also been taken up as can be clearly seen in Fig. 10(c). Note that the amount of the particles internalized in the cytoplasm is adjusted to the individual cellular level and differs significantly from cell to cell. The wide range parallel EELS (WR-PEELS) and ESI Fe elemental studies (Fig. S2†) confirmed that the up taken black objects seen in Figs. 10(a), (b) and (c) have the composition of iron oxide.

To gain a deeper understanding of the endocytosis mechanisms, the particle distribution in a single cell was furthermore investigated. An enlarged image is displayed in Fig. 10(c). It was found that the NPs are endocytosed through both phagocytosis and pinocytosis. The bigger particle aggregates existing outside the cell are phagocytosed via a two step process. Firstly, the aggregates are enclosed by macrophage surface veils outside the cell annotated with "os" in the figure. Secondly, they are internalized into the cell lumen and cellular matrix matter (white arrows). The situation after cytoplasmic uptake (denoted by "is") can also be characterized by the presence of an amorphous medium dense matter. Notably, the cells treat the smaller particles in a different way. Individual to triplex NPs which are in contact with the cell surface are endocytosed by pinocytosis annotated with "pc" in the figure. They can be recognized within the cytoplasm as small envesiculated entities. Interestingly, the amount of single and/or oligomeric NPs taken up by pinocytosis is independent of the particle dose.

2.5 Homogeneous magnetic bioassays in RMF

The PEGylated nanoparticles were labeled with Herceptin antibodies via conjugation chemistry as described in the Supporting Information†. The conjugation scheme is illustrated in Fig. 11. To activate the hydroxyl terminal groups of the PEGylated particles and preparing them for reacting with amine containing Herceptin antibodies, their hydroxyl groups were activated with *N,N'*-Disuccinimidyl carbonate and converted into succinimidyl carbonate groups. The Herceptin antibodies are bound to the particles via amidation facilitated by amine-

Fig. 10 TEM micrographs of in-situ ultrathin sections of the macrophages treated with NP-PEG suspensions in DMEM. Typical views of a single treated macrophage at iron concentrations of (a) $30 \mu\text{gFe mL}^{-1}$ and (b) $0.3 \mu\text{gFe mL}^{-1}$, respectively, and (c) a magnified view of a cell annotated with different endocytosis mechanisms as discussed in the text. N = nucleus and ER = endoplasmic reticulum.

reactive NHS moieties.

Fig. 11 Schematic illustration of labeling of PEGylated nanoparticles with Herceptin antibodies using *N,N'*-Disuccinimidyl carbonate as a homobifunctional NHS ester cross-linker.

The response of magnetic NPs to a RMF results in a phase lag ϕ between the rotating field vector H and the particle magnetic moment m as illustrated in the inset of Fig. 12. The phase lag can be measured magnetically and used for analyzing the particle hydrodynamic properties.⁶ This measurement scheme enables us to differentiate the particles which are bound to target molecules from the not bound ones by measuring the phase lag ϕ change caused by the increasing hydrodynamic size. Ultimately, this allows us to realize sensitive homogeneous bioassays.²⁴

Fig. 12 depicts the measured phase lag of (a) NP-OA, (b) NP-PEG and (c) Herceptin functionalized nanoparticle (NP-Herceptin) suspensions for 1 mT rotating field as a function of frequency. A systematic increase of the phase lag is observed as the particle hydrodynamic size enlarges firstly by the PEGylation and secondly via labeling with Herceptin. By fitting the measured phase lags with the empirical model describing the magnetization dynamics of magnetic nanoparticles in a rotating magnetic field given by Yoshida et al.,²³ the hydrodynamic diameter d_h and its standard deviation σ_h can be determined. Fitting with magnetic moment $m \pm \sigma_m$ calculated from the saturation magnetization M_s (refer to Fig. 8(a)), mean core diameter d_c , dynamic viscosity η , temperature T and k value obtained from ACS results in hydrodynamic diameters of 34 ± 4.6 nm, 47.5 ± 12.3 nm and 69 ± 24.5 nm for NP-OA, NP-PEG and NP-Herceptin NPs, respectively.

The fit of the phase lag spectrum measured on the NP-Herceptin NPs shows a noticeable deviation from the measurement results at the low frequency regime. The observed

Fig. 12 Measured phase lag spectra (markers) of NP-OA (a), NP-PEG (b) and (c) NP-Herceptin suspensions in 1 mT rotating magnetic fields. Parameters of simulated phase lags (lines): (a) $m=1.41\pm0.35$ aA m², $d_h=34\pm4.6$ nm, $\eta=0.56$ mPa s (toluene), $T=297$ K, $k=0.067$, (b) $m=1.29\pm0.31$ aA m², $d_h=47.5\pm12$ nm, $\eta=0.98$ mPa s (water), $T=294$ K, $k=0.066$, (c) $m=1.29\pm0.31$ aA m², $d_h=69\pm24.5$ nm, $\eta=0.93$ mPa s (water), $T=296$ K, $k=0.09$. The inset illustrates the particle phase lag ϕ in RMF. The sample iron concentration was 7, 9.5 and 0.25 mM Fe, respectively.

deviation is presumably due to the presence of a small amount of clusters ($d_h=130$ nm, measured by PCCS) in the sample which are not taken into account in the physical model applied for the fitting. These clusters solely contribute at low frequencies to the sample signal due to their relative slow dynamic response. Although the d_h value of the NP-OA determined by the fitting agrees with the PCCS results but, it appears that by further surface modification (i.e. PEGylation and Herceptin labeling) the d_h value obtained from PCCS and determined by the fitting diverges. It should be noted that PCCS results on suspensions consisting of single and clustered particles are strongly affected by the Rayleigh stray light depending on the sixth power of the hydrodynamic diameter. Admittedly, the functionalization process needs further optimization in order to avoid particle clustering and eventually establish a more sensitive detection method. The described magnetic detection scheme based on the manipulation of 60 nm PEGylated NPs, mainly relaxing via the Brownian relaxation, in the fluxgate-based RMF setup enabled us to monitor the particle phase transfer and functionalization processes and to approximate the change in the particle hydrodynamic diameter after each surface modification step.

To assess the functionality of the NP-Herceptin NPs for recognizing tumor-specific HER2 biomarkers, their phase lag change after adding 5 μ L (0.1 μ g/ μ L) HER2 biomarkers into 150 μ L NP-Herceptin particle suspensions was mea-

sured. The particle phase lag changes noticeably after labeling with HER2 biomarkers, indicating their binding to the NP-Herceptin NPs. As a negative control experiment, HER2 biomarkers were added to the PEGylated NP suspensions and then the particle phase lag was recorded. Notably, the particle phase lag retains the same which means that without having Herceptin receptors on the particle surface, HER2 biomarkers do not link to the PEGylated nanoparticles.

3 Conclusions

In this study, it has been demonstrated that the PEGylation of iron oxide NPs via exploiting nitrodopamine as the anchor is a robust phase transfer strategy to preserve monodispersity and magnetic properties of the synthesized 25 nm iron oxide NPs. The particles are constituted of ferrimagnetic Fe₃O₄ and paramagnetic FeO phases and thereby their saturation magnetization is lower than the values for bulk magnetite. The PEGylated NPs have shown an excellent water stability over a month of being monitored, and revealed no tend to aggregate even at 40°C. No release of inflammatory or anti-inflammatory cytokine in the treated macrophages and a moderate cytotoxic effect was observed even at an iron concentration of 30 μ g_{Fe} mL⁻¹. The particle uptake by macrophages reduced significantly as the iron concentration decreased. It was found that larger clusters are phagocytosed via a two step process whereas individual to triplex NPs are endocytosed by pinocytosis. The presented particle synthesis strategy enabled us to shrink the fraction of the Néel-relaxation-dominated particles to $\approx 7\%$. This feature is considered as a crucial criterion in particle optimization for the magnetic detection schemes based on the Brownian relaxation mechanism. The Herceptin functionalized NPs showed a higher phase lag compared to the PEGylated ones, indicating the linkage of Herceptin antibodies to the particle surface. The results showed that the monodisperse PEGylated NPs with a comparatively small hydrodynamic size of 60 nm are a potent tracer for magnetic detection schemes based on the particle manipulation in dynamic magnetic fields (e.g. RMF and ACS). Besides these NPs are capable of being bound to small target molecules owing to their small size, making them preferable to the commercially available multi-core nanoparticles. Note that in such magnetic bioassays, the functionality of bound Herceptin antibodies should be preserved during the conjugation reaction, otherwise the bioassay will fail. Further studies to examine the reactivity of antibodies after binding to nanoparticles for recognition of HER2 biomarkers are in progress.

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