Design of triphasic poly(lactic-co-glycolic acid) nanoparticles containing a perfluorocarbon phase for biomedical applications†

Edyta Swider, †a Alexander H. J. Staal,a N. Koen van Riessen,a Linsey Jacobs,a Paul B. White,b Remco Fokkink,c Geert-Jan Janssen,e Eric van Dinther,a Carl G. Figdor, †a I. Jolanda M. de Vries, cad Olga Koshkina c, †a and Mangala Srinivas b, †b

Poly(lactic-co-glycolic acid) (PLGA) particles are very widely used, particularly for drug delivery, including commercial clinical formulations. Adding perfluorocarbon (PFC) enables in vivo imaging and quantification of the PLGA particles through 19F NMR, MRS or MRI. PFCs are both hydrophobic and lipophobic at the same time. This property makes their encapsulation in particles challenging, as it requires the addition of a third immiscible phase during the emulsification process. Here we explore how different parameters affect the miniemulsion formation of particles loaded with perfluoro-15-crown-5-ether (PFCE). By changing the concentration of surfactant and type of solvent, we were able to control the radius of synthesized particles, between 85–200 nm. We assessed stability and release from the particles at different pH values, showing that hydrophobic agents are released from the particles by diffusion rather than degradation. With cell experiments, we show that primary human dendritic cells take up the particles without any apparent effect, including on cell migration. In summary, the control of synthesis conditions leads to particles with sufficient PFCE encapsulation, which are suitable for drug loading and cell labeling, and do not affect cell viability or functionality. Finally, these nanoparticles can be produced at GMP-grade for clinical use.

Introduction

Recent developments in the field on nanomedicine have focused on multifunctional theranostic devices. Such theranostic devices, encapsulating both a contrast agent for imaging, e.g. magnetic resonance imaging (MRI), and a drug for therapy, allow not only for the delivery of a therapeutic compound to the site of interest, but also give the ability to visualize and monitor therapy and progression of the disease.† For these purposes, the use of biodegradable polymeric particles is of great interest due to their ease of processing, biological stability, low toxicity, and effectiveness as delivery agents. Poly(lactic-co-glycolic acid) (PLGA) is particularly attractive for the synthesis of nanoparticles, as PLGA is biocompatible, biodegradable, and shows long-term storage stability.‡ PLGA nanoparticles can be modified, for example with contrast agents and drugs, by either covalent coupling or non-covalent loading using the hydrophobic effect.‡ † PLGA protects the drug from fast degradation and the release time of the encapsulated cargo from the particles can be modulated by altering the synthesis parameters and polymer composition.‡

Loading the particles with liquid perfluorocarbons (PFCs) for fluorine (19F) MRI is advantageous for biomedical use, as it can be used for in vivo non-invasive imaging. Fluorine is essentially absent from biological tissues, therefore, 19F MRI results in high specificity of images and moreover, enables the quantification of the signal, including determination of 19F content and particularly quantification of 19F-labeled cells directly from image data.§ § PFCs are simultaneously hydrophobic and
liphobic synthetic organic compounds with a high payload of fluorine atoms,\textsuperscript{10} with a long history of clinical application, initially as blood substitutes and more recently for imaging applications.\textsuperscript{11,12} To overcome the immiscibility of PFCs, liquid PFCs are usually delivered as surfactant-stabilized PFC emulsions for \textsuperscript{19}F MRI. However, the colloidal stability of PFC emulsions is relatively low, especially at high concentrations required for cells loading, which further may lead to accumulation in organs and poor biodistribution.\textsuperscript{9,13-16} The stability can be improved with the use of phospholipids or poloxamers, however both types of surfactants exhibit some disadvantages, e.g. they are prone to hydrolysis and oxidation (phospholipids) or they tend to form highly viscous dispersions (poloxamers).\textsuperscript{17}

Encapsulation of PFCs into polymeric nanoparticles is relatively new to the field and has some specific advantages, particularly for cell loading and imaging. Here, perfluoro-15-crown-5-ether (PFCE) is of special interest, as it is chemically and biologically inert and consists of 20 chemically equivalent \textsuperscript{19}F nuclei, leading to a singlet in NMR spectra, which is an important factor for quantification and MR imaging.\textsuperscript{18,19} PFCE-loaded PLGA particles can be synthesized using a miniemulsion formulation technique (Fig. 1).\textsuperscript{5} It has been previously shown that parameters such as type of solvent and surfactant, and concentration of surfactant and polymer can influence the characteristics of PLGA nanoparticles prepared by the solvent evaporation method.\textsuperscript{20}

Aiming at different applications, such as cell tracking and imaging of drug delivery, it is important to control the properties of PLGA–PFC particles, including diameter, diameter distribution, fluorine content, and encapsulation efficiency of hydrophobic drug or other agents. Particle size and size distribution influence the in vivo distribution, cell uptake, biological fate, toxicity and the targeting ability. For example, Waiczies \textit{et al.} reported on fluorine-rich emulsion particles, where larger particles (280 nm in radius) were more readily taken up by dendritic cells (DCs) and promoted the DCs maturation process.\textsuperscript{21} These particle characteristics can also affect drug loading, drug release, and stability of particles.\textsuperscript{22} However, systematic study of perfluorocarbon-loaded PLGA nanoparticles have not been reported thus far. Hence, there is little prior work describing a systematic study of triphasic PLGA–PFC systems, although these have been shown to have a very high loading and excellent stability.\textsuperscript{18} In this work, we explore the influence of different synthesis parameters on the radius, homogeneity, stability and encapsulation efficiency of PLGA–PFCE nanoparticles and the influence of the PFC phase on particle characteristics. We focus only on PLGA–PFCE nanoparticles, which are very different from PLGA particles that are extensively described in the literature. We also investigate the impact of PFC-encapsulation on the release characteristics of PLGA particles. For this, we introduce nile blue dye into the nanoparticles, as a model hydrophobic drug. Finally, we compare the cellular uptake of different particles in primary human monocyte-derived dendritic cells (moDCs), and the effects of uptake on cell viability and functionality. Overall, the objective of the present study is to show how different synthesis parameters influence the synthesis of PLGA particles with a third PFC phase, with a view to biomedical applications.

### Results and discussion

#### Change in surfactant concentration and type of solvent influences the final properties of particles

The main parameters, which affect the synthesis of PLGA particles are type and concentration of surfactant, concentration of polymer, and type of solvent for the organic phase (Scheme 1).\textsuperscript{1,21,24-26}

Loading the particles with PFC alters the properties of miniemulsions, as PFCs form their own phase in solutions. Among different surfactants, polyvinyl alcohol (PVA) with low molecular weight (10 kDa) resulted in nanoparticles with a small hydrodynamic radius ($R_h$), and high PFCE loading. Thus, by reducing the concentration of surfactant, in this case PVA, which is perhaps the most often used surfactant for synthesis of PLGA particles, we could obtain particles with a larger size. This result is similar to PLGA particles without PFC (Fig. 2).\textsuperscript{26,27}

However, the polydispersity of particles also increased, while the encapsulation yield of PFCE dropped, perhaps because at lower concentrations of surfactant, stabilization of the PFCE-droplets was not achieved. In a miniemulsion, diameter and diameter distribution of droplets directly depend on the

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**Fig. 1** Schematic representation of miniemulsion formulation method. PLGA and PFCE are added to surfactant solution under sonication, followed by solvent evaporation. Next, particles are washed, freeze-dried, and collected as a dry powder.
concentration of surfactant. A higher concentration of surfactant in the aqueous phase (in this case 2.0 wt%) leads to sufficient coverage of the interface between droplet and aqueous phase by surfactant, which stabilizes the emulsion droplets and prevents the coalescence. Hence, a higher concentration of surfactant results in more homogenous particles with a narrower size distribution. This effect is reflected in lower PDI values (Fig. S1†), as well as in lower standard deviation between measurements. Thus, particles prepared with 2 wt% of surfactant have lower PDI values and the diameter measurements also display better reproducibility than at lower concentration of surfactant. Interestingly, several other surfactants that are commonly used in synthesis of PLGA particles without PFCE,27,28 were not able to stabilize particles when the PFCE phase was added (Table S1†).

Furthermore, increasing concentration of polymer, while keeping the volume of organic phase and other parameters constant, resulted in larger particles with lower PFCE loading (Fig. 2B). Typically, increasing concentration of polymer leads to the formation of bigger droplets due to increased viscosity of the organic phase.20,29,30 The total amount of obtained nanoparticle yield was slightly higher when we increased the concentration of PLGA, while the overall amount of PFCE in the whole batch was at around 20 mg for all three concentrations of polymer (Table 1). Finally, the type of solvent used for miniemulsion process did not affect the properties of the final particles. Thus, using chloroform or ethyl acetate, which are both higher polarity solvents resulted in similar particles as with dichloromethane (DCM) (Table S2†).

Along with the miniemulsion technique, nanoprecipitation is another common technique for the synthesis of PLGA nanoparticles. We investigated nanoprecipitation to better understand the formation of nanoparticles. As nanoprecipitation does not involve the use of shear forces; it is not suitable for the encapsulation of PFCE. However, we modified a typical nanoprecipitation procedure by applying sonication during the addition of the organic to aqueous phase; this resulted in increased size and polydispersity of nanoparticles, and low PFCE encapsulation (Table S2†).

Combination of DCM with other solvents with different polarities influences the size of particles

Interestingly, in contrast to replacing DCM with another water immiscible solvent (Table S2†), mixing DCM with solvents of

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Combination of DCM with other solvents with different polarities influences the size of particles

Interestingly, in contrast to replacing DCM with another water immiscible solvent (Table S2†), mixing DCM with solvents of
higher or lower polarity, while keeping the concentration of surfactant and polymer constant (2 and 1.75 wt% respectively), resulted in altered size of particles, with efficient encapsulation of PFCE (Table S3†). Thus, the combination of DCM with the higher polarity solvent acetonitrile (MeCN, relative polarity 0.460) or lower relative polarity solvent toluene (relative polarity 0.099) resulted in smaller or bigger size respectively, according to Transmission Electron Microscopy (TEM) (Fig. 3B and C). The differences between solvents result from the interfacial tension between aqueous and organic phase. The interfacial tension between aqueous and organic phase is higher for solvents with lower polarity, such as DCM + toluene mixture.31 Bigger droplets have lower surface-to-volume ratio. Therefore, for oil-in-water miniemulsion the formation of bigger droplets is facilitated, when solvents with lower polarity are used for dispersed phase, compared to solvents with higher polarity.31 An additional reason for the smaller size of particles prepared with DCM + MeCN mixture could be a diffusion of water miscible MeCN into aqueous phase directly after the formation of the droplets. This diffusion results in faster shrinking of the droplets and thus in smaller size of the final particles. However, further experiments are necessary to confirm this mechanism.

TEM data analysis of these nanoparticles differed from the results of Dynamic Light Scattering (DLS) (Table S3†). TEM results showed a bimodal size distribution (151 nm and 85 nm fractions) of nanoparticles prepared with DCM + toluene as solvent (Fig. 3C), while DLS measurements of these particles showed a radius of 195 nm. These DLS measurements were

<table>
<thead>
<tr>
<th>Nanoparticle (solvent type)</th>
<th>$R$ (nm) ± SD (cryoSEM)</th>
<th>$R_h$ ± SD (DLS at 173°)</th>
<th>PFCE encapsulation (wt%)</th>
<th>Dye concentration μg ml$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>DCM</td>
<td>120 ± 20</td>
<td>93 ± 5</td>
<td>22</td>
<td>8</td>
</tr>
<tr>
<td>DCM + MeCN</td>
<td>90 ± 20</td>
<td>85 ± 2</td>
<td>19</td>
<td>6</td>
</tr>
<tr>
<td>DCM + toluene</td>
<td>253 ± 62, 110 ± 25</td>
<td>117 ± 32</td>
<td>19</td>
<td>12</td>
</tr>
</tbody>
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Table 1 Characterization of nanoparticles prepared with combination of different solvents
done with backscatter detection at 173°, which is less sensitive to the presence of larger particles, as larger particles primarily scatter light at forward angles. For particles larger than \( \lambda/20 \) (\( \lambda \) is wavelength of the laser) the normalized single particle scattering intensity the particle form factor \( P(q) \) becomes dependent on scattering angle, represented by the scattering vector \( q \). Thus, the values of the diffusion coefficient at single measurement angles are apparent values. Therefore, to obtain the absolute 2-average of the diffusion coefficient for calculation of absolute hydrodynamic radius, the apparent values of the diffusion coefficient need to be extrapolated \( q \to 0 \), which corresponds to \( P(q) = 1^{1/2} \). To investigate this discrepancy between TEM and single-angle DLS, we characterized the particles with multi-angle DLS.

Fig. 3D–F shows the angular dependency of the inverse hydrodynamic radius \( 1/R_h \), which was obtained from apparent diffusion coefficients with the Stokes–Einstein equation. Extrapolation \( q \to 0 \) results in inverse 2-average of radii \( <R_h>^{1/2} \) of 182, 163 and 308 nm for DCM, DCM + MeCN and DCM + toluene nanoparticles respectively, with the \( \mu_2 \) values of second cumulant from cumulant analysis of autocorrelation function at \( \theta = 90° \) of 0.13, 0.08 and 0.27 (Fig. 3D–F). Typically, particles with \( \mu_2 \) values smaller than 0.05 are considered as monodisperse, while values higher than 0.2 are described as polydisperse. As the size distribution of particle seems to be non-Gaussian according to TEM, especially for toluene particles, we did not recalculate \( \mu_2 \) values into PDI value. Further analysis of angular dependency of DCM + toluene particles indicates the presence of two size fractions, which were also visualized by TEM. Thus, when only apparent values from \( \theta = 30°−70° \) are used, the resulting \( <R_h>^{1/2} = 510 \) nm, while extrapolation of bigger angles results in \( <R_h>^{1/2} = 182 \) nm. Generally, the hydrodynamic radii obtained from DLS are bigger than the radii from TEM, indicating that particles shrink when dried for TEM. Especially, in the case of DCM + MeCN particles this effect seems to be quite pronounced, as the hydrodynamic radius is three times higher than the TEM-determined radius. To study this behaviour further we measured cryogenic Scanning Electron Microscopy (cryoSEM), which enables imaging of particles in a solution-like state. CryoSEM demonstrates that the majority of DCM + MeCN particles have an approximate radius around 65–90 nm, however, some particles have a radius around 125–175 nm. With LS, only this fraction of bigger particles could be detected. The results from cryoSEM on DCM + toluene particles confirm the presence of both sizes 90–130 nm and 180–230 nm. We also observed two fractions of particles in multi-angle LS as presented in Fig. 3C. Thus, cryoSEM confirms the results, which were obtained with LS.

The size of particles affects the release rate of hydrophobic agent

The release rate is a very important parameter when developing systems suitable for different biomedical applications, as it determines whether the nanoparticles will be able to reach the site of interest, before significant degradation and loss of active component has occurred. Factors influencing the release rate include e.g. particle radius and pH of the environment. The pH of the medium has a significant influence on PLGA degradation, as a lower pH increases the degradation rate of polymer chains.

To study the effect of size and PFCE-loading on release, we synthesized particles with the addition of fluorescent dye, Nile blue, as a model compound for hydrophobic drugs, such as paclitaxel, which have previously been encapsulated in PLGA nanoparticles. Concentration of dye varied between different nanoparticles and increased with an increase in nanoparticle radius (Table 1), as determined by ultraviolet/visible (UV/vis) spectroscopy. The particles were incubated at 37 °C in solutions with pH values of 4.0, 5.5 and 8.5 to compare the release in acidic and alkaline conditions. These pH values were chosen based on the pH of different subcellular compartments, namely lysosomes, endosomes and mitochondria respectively. A study by Panyam et al. showed that PLGA nanoparticles of approximate mean radius of 0.05 μm, incubated at 37 °C in pH 7.4 maintained their structural integrity for up to 20–30 days, followed by the formation of pores, deformation and fusion of particles. Thus, we expect PLGA–PFCE particles to remain stable after in vivo injection, until they are taken up the immune cells and reach the targeted site.

The percentage of released dye was calculated based on the UV/vis results (Fig. 4A–C). Mid-size and bigger particles, which were synthesized with DCM (Fig. 4A) and DCM + toluene (Fig. 4C), showed higher burst release of encapsulated dye at pH 4 after 6 hours. Even at later time points the release remained higher than at higher pH. In case of DCM particles, the release at pH 8.5 seems gradual, while at pH 5.5 faster release was observed within first two days and saturated afterwards. Larger particles display faster release within first two days, followed by saturation of both release curves. The release of dye from smaller particles was generally higher compared to both other samples. This trend can be explained by the fact that smaller particles have a larger surface area to volume ratio. With a larger surface area, water has better accessibility to enter the particles, resulting in an increase of degradation and release of the encapsulated compound. In the case of smaller particles, the release at pH 4 was also faster than at higher pH, although the difference was less pronounced than with larger particles.

It has been previously demonstrated that smaller nanoparticles display higher polymer degradation and release rates during the initial phase compared to larger particles. In our experiment, we also observed faster release of dye from DCM + MeCN particles (Fig. 4B) during the first 4 days of incubation when compared to DCM + toluene particles with larger radius (Fig. 4C). This trend can be explained by the fact that smaller particles have a larger surface area to volume ratio; with a larger surface area, water has better accessibility can more readily enter the particle, which further results in increased release of the encapsulated compound. While replicating the pH of various cell compartments, our model/assay lacks the presence of various enzymes that can influence the release.

To detect any changes in the average radius of nanoparticles and PDI upon incubation, we measured samples at various time points using DLS. The results are presented in Fig. 4D–F, and...
We observed a slow gradual decrease in particle radius (Fig. 4D–E) with an average reduction of about 10 nm within 24 days, for particles prepared with DCM and DCM + MeCN solvents (93 to 80 nm and 85 to 75 nm respectively). No significant changes in the radius of particles formulated with DCM + toluene mixture were detected (Fig. 4F).

In general, the size of particles decreased only slightly during the whole period, indicating that diffusion of the dye rather than particle degradation caused release. Note, however, that the dye itself has an adsorption peak close to the wavelength of the laser and could affect size determination. The increase in radius of DCM-toluene particles at 0 h time point could be due to agglomeration of the sample. However, the backscattering set up, which we used for size control during the release experiment, was not able to detect the fraction of bigger size fraction, which we detected with TEM and with multi-angle DLS. With smaller particles, a low fraction of larger particles affected determination of size, as shown in the previous section. Therefore, the limitations of a backscattering set up should be taken into account when looking at the results of these size measurements.

Overall, we found that PFCE-loaded particles show similar trends in release as PLGA nanoparticles without PFCE. In literature, PLGA particles do not show significant changes in size until up to 14 days of incubation in ref. 7, 37 and 39. For MRI applications, PFCs are usually used as an emulsion, which are stabilized by surfactants. The colloidal stability of these emulsions is usually low, in a range of hours to days especially at higher concentrations, as the stabilization of the PFC phase is a challenge due to solubility properties of PFCs. By encapsulation of this third PFC phase in the PLGA matrix, we obtained particles that were stable for at least 21 days at 37 °C, which is a significant improvement over many formulations of PFC emulsions. Furthermore, the stability of the particles, which were lyophilized and stored in a freezer, is at least 6 months.

To assess the influence of the third phase on the encapsulation of the hydrophobic agent, we synthesized particles encapsulating Nile blue, and with or without the addition of PFCE. The highest encapsulation of Nile blue was achieved with nanoparticles prepared with the use of DCM as solvent, both with and without the addition of PFCE, as presented in the Fig. S4†. Furthermore, the difference in Nile blue encapsulation between these particles was minimal. Thus, we conclude that the incorporation of even relatively large amounts of PFC does not significantly affect the encapsulation and release of hydrophobic agents from the PLGA particles.

Nanoparticles are suitable for the loading of cells

To study the uptake of PFCE–PLGA nanoparticles, we loaded human moDCs, as used in clinical trials, with the same particles that were used for the dye release experiment, followed by either 1 or 24 hours of incubation at 37 °C, depending on the assay. We chose DCs as a model for a therapeutic cell type, due to their use in cancer immunotherapy a fast-growing field. We have previously shown that nanoparticle-loaded DCs can be imaged in vivo, using 19F MRI. However, here we focused on the chemical optimization of the particles and ensuring that they are located intracellularly.

We used confocal microscopy to determine the intracellular presence of PFCE–PLGA particles in moDCs. After incubation with particles for 24 hours, cells were stained for the early
endosomal marker EEA1 and the late endosomal/lysosomal marker LAMP1 to study the potential intracellular localization of nanoparticles. As presented in Fig. 5A and S3A and B†, fluorescent signal from the particles (arrows) partially overlaps with the EEA1 signal. More co-localization of nanoparticles was observed with the LAMP1 fluorescent signal. Part of the fluorescent signal coming from the nanoparticles did not localize with neither EEA1 nor LAMP1 signal. As explained in a previous study, lack of co-localization could mean that some nanoparticles escape from the endosomal pathway. Another explanation could be that nile blue leaked out of the particles resulting in the signal detection outside of the endosomes or lysosomes.

Size of particles is an important factor in biodistribution, and in obtaining therapeutic effectiveness. For example, PLGA nanoparticles smaller than 50 nm in radius have lower plasma protein adsorption on their surface and lower hepatic filtration. It has been shown that larger particles can be taken up more efficiently by phagocytic cells than particles of smaller radius. Particles larger than 250 nm in radius are typically taken up via phagocytosis, while the uptake of smaller nanoparticles occurs via endocytosis. Other particle characteristics which can affect the cellular uptake include charge, shape and surface topology. Here, we show that all three sizes of particle were readily taken up by the cells. However, uptake may differ with cell type, including other DC subsets, macrophages or non-phagocytic cells. Thus, synthesizing particles of different sizes is crucial, as it gives the ability to target different types of cells.

One of the applications of PFCE–PLGA nanoparticles is their use as cell tracking agents for 19F MRI. To investigate whether nanoparticle-loaded cells can be detected with 19F MRI, we first incubated moDCs in the presence of DCM, DCM + MeCN or DCM + toluene particles and then imaged them after 3 days of incubation. As shown in Fig. 5B, we were able to detect the fluorine signal from all three samples, using a biocompatible imaging sequence and within a reasonable imaging time (15 minutes). The differences in the signal intensity are due to different encapsulation of PFCE among the nanoparticles used for this assay. To investigate whether uptake affects cell functionality, we studied cell migration; both the velocity and the distance covered by the labelled cells. The results of the

![Fig. 5](image)

**Fig. 5** Effect of nanoparticles on monocyte-derived DCs. (A) Confocal microscope images of nanoparticle uptake by DCs. Fluorescent signal coming from the nanoparticles partially overlaps with the signal of the early endosomal marker EEA1. Higher colocalization of nanoparticles was observed with the signal of late endosomal/lysosomal marker LAMP1. (B) 1H/19F MRI image showing the signal of 5 × 10⁶ cells loaded with different nanoparticles. Here, PFCE–PLGA particles, at 10 mg ml⁻¹ concentration, were used as a reference. (C) Nanoparticles do not affect the migration of dendritic cells. The graph represents average velocity of unloaded cells and cells loaded with nanoparticles. (D) The graph shows the viability of cells after incubation with various nanoparticles for 72 hours. No significant influence on cell viability was observed. Error bars represent the standard deviation (n = 3).
measurements of average cell velocity are presented in Fig. 5C. We observed an increase in velocity of cells incubated with particles when compared to control cells (in red), which corresponds to the fact that nanoparticle uptake can induce the migration of DCs. The mean velocity was 0.56 ± 0.22, 0.79 ± 0.34, 1.19 ± 0.53 and 1.11 ± 0.41 μm min⁻¹ for unloaded controls, DCM nanoparticle, DCM + MeCN nanoparticle or DCM + toluene nanoparticle-loaded cells respectively. The trajectory of migrating cells for all four conditions is presented in Fig. S5.7 We further confirmed the cell viability using standard viability assay (Fig. 5D). Our results, show that the particles are suitable for cell labelling and imaging with ¹⁹F MRI, and have no significant effect on viability or functionality.

Conclusions

The size of particles, size distribution and degradation are important factors for various biomedical applications, including controlled delivery of drugs, drug release and cell uptake. Control of these properties of nanoparticles is crucial for optimal particle performance. In this study, we have shown that a narrow size distribution and high ¹⁹F loading in PFCE–PLGA nanoparticles can be achieved by using an optimal concentration of surfactant and the right solvent. We also showed that nanoparticle size has a strong influence on the encapsulation and release of the encapsulated cargo. Here, we used nile blue as a model for a hydrophobic agent, such as paclitaxel. PFCE–PLGA nanoparticles can be further adapted for targeting by the addition of targeting ligands. The use of such multifunctional nanoparticles for biomedical applications in cancer or other diseases has great potential for diagnosis and targeted treatment, such as targeted delivery of hydrophobic anticancer drug combined with quantitative ¹⁹F MRI, or cell tracking. In particular, ¹⁹F MRI is a quantitative technique that is not limited by penetration depth in vivo; therefore, it would be possible to use non-invasive in vivo imaging to quantify drug delivery using these nanoparticles. Finally, we have shown that this method of preparation of PLGA particles is robust, and not significantly affected even by the addition of large amounts of a third immiscible phase, the PFC. Overall, our results could provide a guideline in the rational design of polymer-based particles loaded with a third PFC phase for imaging and drug delivery, where size, encapsulation efficiency and release can be controlled.

Materials and methods

Materials

Poly[(L,L-lactide-co-glycolide)] (PLGA) resomer RG 502H, lactide : glycolide molar ratio 50 : 50 was obtained from Evonik Industries AG, Essen, Germany. Solvents: dichloromethane (DCM), chloroform (Merck, Darmstadt, Germany), ethyl acetate (AcOEt), acetone, acetonitrile (MeCN) (all from VWR, Netherlands), tetrahydrofuran (THF) (Sigma-Aldrich, St. Louis, MO, USA). Perfluoro-15-crown-5-ether (PFCE) was purchased from Bracco, Konstanz, Germany. Surfactants: polyvinyl alcohol (PVA, 9000–10 000 M₉₀, 80% hydrolyzed), pluronic F68, sodium cholate, polyvinylpyrrolidone (PVP) were purchased from Sigma-Aldrich St. Louis, MO, USA, and Tween20 from Merck, Darmstadt, Germany. Some nanoparticles were prepared with Nile blue dye (Acros Organics, Belgium) For cell culture X-VIVO 15 medium (Lonza, Belgium) or RPMI 1640 (Gibco, Thermo Fisher, The Netherlands) was used. Phosphate-buffer saline (PBS) (Braun, Germany) was used to resuspend nanoparticles for cell experiments and for cell washing.

Particle synthesis

Particles were prepared using a miniemulsion synthesis technique. Briefly, PLGA (100–200 mg), was dissolved in 3 ml of various solvents, followed by the addition of 900 μL PFCE and 1.8 ml Prohance. For dye release and cellular uptake experiments, Nile blue dye (0.2 mg) was also included in the organic phase. The organic phase was added to 25.5 g of aqueous solution of surfactant (0.4–2 wt%) and emulsified for 3 minutes with the use of probe sonicator (Sonifier 250, microtip 6.3 mm, Branson Sonic Power, St. Louis, USA) at 40% amplitude. Sonication was followed by overnight evaporation of solvent at room temperature. Next, the particles were collected by centrifugation (15 000 rpm, 20 minutes), washed several times with deionized water, and then freeze-dried. Several parameters tested in this protocol, including: the type of surfactant, the type of solvent, polymer concentration (3.3, 5, 6.6 wt%) and surfactant concentration (0.4, 1.2, 2 wt%). All particle characterization was done on the freeze-dried samples which.

Particle characterization

Dynamic light scattering was measured at Malvern zeta sizer NS nano (Malvern, Worcestershire, United Kingdom), using disposable ZEN0040 cuvettes, at concentrations of 0.1 mg ml⁻¹. Data evaluation was done with Malvern software (Zetasizer software, Ink) using cumulant fitting. The values are the intensity mean of 4 independent measurements.

Multi-angle LS measurements were performed using an ALV7004 correlator, ALV/LSE-5004 Goniometer, ALV/Dual High QE APD detector unit with fibre splitting device with a set-up of 2 off detection system and a Uniphase Model 1145P He–Ne Laser. The laser wavelength and power were 632.8 nm and 22 mW, respectively and the temperature was controlled by a Julabo CF41 thermostatic bath. Water for dilutions was filtered with 0.45 μm hydrophilic filters. The data analysis was done with HDRC software, which was kindly provided by Prof. Manfred Schmidt, University of Mainz, Germany. The apparent radii of hydrodynamic radii at different angles were calculated using from a biexponential fitting of autocorrelation function, as it provides a good approximation to calculate the size of monomodal samples with a non-monodisperse size distribution. The absolute inverse ζ-averages of hydrodynamic radii were obtained by extrapolating $g \rightarrow 0$. The second cumulant values $\mu_2$ were obtained from cumulant fitting at $\theta = 90^\circ$. The $\mu_2$ value is the second cumulant, obtained from the cumulant analysis of autocorrelation function and provides a quantitative
Peripheral blood mononuclear cells (PBMCs) were isolated from:

Cell culture and labeling

- Vis spectrophotometer, JASCO Benelux, B.V.). Obtained data of the supernatant and measuring its absorption (JASCO V-630 UV-vis spectrophotometer).
- Additionally, dye release from nanoparticles was tested at each concentration 10 mg ml⁻¹ (0.3% PVA solution with either 1 M HCl or NaOH) of 4.0, 5.5, 8.5, and granulocyte-monocyte colony stimulating factor (450 U ml⁻¹) to obtain immature dendritic cells (DCs).
- 3 cells were harvested, counted and labeled with PLGA nanoparticles (resuspended in PBS shortly prior the labelling) at concentration 2 mg of nanoparticles/1 x 10⁶ cells and incubated at 37 °C for 1 and 24 h for confocal microscopy, 24 h for cell migration, 72 h for viability assay and 72 h for MRI imaging.

Cell staining and confocal microscopy

- The uptake of various nanoparticles containing nile blue was confirmed with confocal microscopy. Day 3-moDCs were incubated at 37 °C for 1 and 24 h on glass coverslips (20 000 cells per coverslip). At each time point the excess of the label was removed, the coverslips with cells were washed gently with PBS, and then the cells were fixed with 2% paraformaldehyde (PFA) for further testing with confocal microscopy.
- Labelled cells on coverslips were permeabilized in CLSM buffer + 0.1% Saponin, stained first with LAMP-1 or EEA1-specific primary antibody (Ab), followed by staining with isotype-specific AlexaFluor conjugated secondary Ab for intracellular compartments, phalloidin-488 for cell membrane, and DAPI for nucleus.
- Stained cells were then imaged with Olympus FV1000 Confocal Laser Scanning Microscope. Acquired images were then processed in the ImageJ program.

Cell migration

- To study cell migration and velocity, nanoparticles were resuspended in PBS shortly before the labelling and added to day-3 moDCs at concentration 2 mg of nanoparticles per 1 x 10⁶ cells. Cells with nanoparticles were then added to 24 well plates and imaged for 24 hours using Okolab 2.0 microscope (Nikon Diaphot 300 with Hamamatsu C8484-05 G CCD Camera) with Okolab 4 well CO₂ stage incubator and Okolab 2D time lapse software. Acquired images were then processed using ImageJ program to measure the distance and velocity.

Cell viability

- To investigate the influence of labelling on cells viability with performed an MTT assay. For this, 0.5 x 10⁶ cells were incubated in the presence of DCM, DCM + MeCN or DCM + toluene nanoparticles for 72 hours. Each condition was done in triplicate. After incubation, the excess of the label was removed by gentle washing with PBS. Next, the cells were collected and placed in flat-bottom 96 well plates, washed twice in PBS (100 µl per well) with centrifugation for 2 min between each wash. Next, 60 µl RPMI medium with 10 µl MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (concentration 4 mg ml⁻¹) was added to each well, followed by 30 minutes incubation at 37 °C. After incubation, the plate was centrifuged for 2 min and 100 µl of lysis buffer (isopropanol, 10% SDS, 2 N HCL, deionized water) was added to each well, and the plate was incubated for 15 minutes in dark at room temperature. Before the
measurements, samples were resuspended to remove any precipitate of crystals. The plate was measured with iMark™ microplate reader (Bio-Rad, The Netherlands) at 595 nm. Cells not loaded with nanoparticles were used as negative control and cells treated with 0.2% Triton X-100 were used a positive control.

19F Magnetic resonance imaging
19F MRI was performed on a pre-clinical 11.7 T MRI scanner (Biospec 117/16, 500 MHz, Bruker, Ettlingen, Germany). For in vitro imaging, 5 × 10^6 dendritic cells were co-incubated with particles at a concentration of 10 mg ml^-1. Nanoparticle loaded cells were extensively washed to remove excess label, before being placed in tubes, left to sediment and imaged with the zero echo time (ZTE) sequence with the following parameters: TR = 2 ms, flip angle 4°, matrix size = 32 isotropic voxels, FOV = 60 × 60 × 60 mm, (voxel size = 1.87 mm), number of averages = 128, scan time = 15 minutes. Sedimented particles (5 mg in 5 μl) in PBS were used as the reference.

Author contributions
The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Conflicts of interest
There are no conflicts of interest to declare.

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