Biocompatible Glyconanoparticles by Grafting of Sphorolipid Monolayers on Monodisperse Iron Oxide Nanoparticles

Andrea Lassenberger, Andrea Scheberl, Krishna Chaithanya Batchu, Viviana Cristiglio, Isabelle Grillo, Daniel Hermida Merino, Erik Reimhult, Niki Baccile

► To cite this version:

Andrea Lassenberger, Andrea Scheberl, Krishna Chaithanya Batchu, Viviana Cristiglio, Isabelle Grillo, et al.. Biocompatible Glyconanoparticles by Grafting of Sphorolipid Monolayers on Monodisperse Iron Oxide Nanoparticles. ACS Applied Bio Materials, 2019, 2 (7), pp.3095-3107. 10.1021/ac-sabm.9b00427 . hal-02164319

HAL Id: hal-02164319

<https://hal.sorbonne-universite.fr/hal-02164319>

Submitted on 25 Jun 2019

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

This document is confidential and is proprietary to the American Chemical Society and its authors. Do not copy or disclose without written permission. If you have received this item in error, notify the sender and delete all copies.

**Biocompatible Glyconanoparticles by Grafting of
Sophorolipid Monolayers on Monodisperse Iron Oxide
Nanoparticles**

Journal:	<i>ACS Applied Bio Materials</i>
Manuscript ID	mt-2019-004275.R2
Manuscript Type:	Article
Date Submitted by the Author:	14-Jun-2019
Complete List of Authors:	Lassenberger, Andrea; Institut Laue-Langevin, Scheberl, Andrea; University of Natural Resources and Life Sciences Batchu, Krishna; Institut Laue-Langevin Cristiglio, Viviana; Institut Laue-Langevin, Grillo, Isabelle; Institut Laue-Langevin, Large Scale Structures Group Hermida-Merino, Daniel; Netherlands Organization for Scientific Research, Chemistry Reimhult, Erik; University of Natural Resources and Life Sciences Vienna, Department of Nanobiotechnology Baccile, Niki; CNRS-UPMC, LCMCP UPMC

SCHOLARONE™
Manuscripts

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Biocompatible Glyconanoparticles by Grafting of Sophorolipid Monolayers on Monodisperse Iron Oxide Nanoparticles

Andrea Lassenberger,^{a,b} Andrea Scheberl,^a Krishna Chaithanya Batchu,^b Viviana Cristiglio,^b Isabelle Grillo,^b Daniel Hermida Merino,^c Erik Reimhult,^{a,} Niki Baccile^{d,*}*

^aUniversity of Natural Resources and Life Sciences Vienna, Institute for Biologically
inspired materials, Department of Nanobiotechnology, Muthgasse 11/II, 1190 Vienna,
Austria

*erik.reimhult@boku.ac.at

^bInstitut Laue-Langevin, 71 Avenue des Martyrs, 38042 Grenoble Cedex 9, France

^cESRF – The European Synchrotron, 71 Avenue des Martyrs, 38042 Grenoble Cedex 9,
France

^dSorbonne Université, Centre National de la Recherche Scientifique, Laboratoire de

Chimie de la Matière Condensée de Paris, LCMCP, F-75005 Paris, France

*niki.baccile@upmc.fr

KEYWORDS: sophorolipid, glyconanoparticle, nanoparticle surface functionalization,

small angle neutron scattering, small angle X-ray scattering, lipid shell, iron oxide

nanoparticle, core-shell nanoparticle, colloidal stability

ABSTRACT. This work presents synthesis and characterization of sophorolipid-coated

monodisperse iron oxide nanoparticles. Sophorolipids are biological glycosylated

amphiphiles produced by the yeast *S. bombicola*. In their open acidic form, sophorolipids

have been used as surface stabilizing agent for metal and metal oxide nanoparticles but

with poor control over size and structural properties. In this work, the COOH function of

sophorolipids (SL) was modified with nitrodopamine (NDA), a catechol known for its high

affinity to iron ions. The resulting new form of sophorolipid-nitrodopamide (SL-NDA) was

1
2
3 used as surface ligand for monodisperse iron oxide nanoparticles. We show, by a
4
5
6
7 combination of thermogravimetric analysis and small angle X-ray and neutron scattering,
8
9
10 that iron oxide nanoparticles (IONP) are stabilized by a single, high-density SL-NDA layer,
11
12
13
14 which results in excellent colloidal stability under biologically relevant conditions such as
15
16
17 high protein and salt concentration. The IONP grafted with SL-NDA showed negligible
18
19
20 uptake and no cytotoxicity tested on two representative cell lines. Thus, they reveal the
21
22
23 potential of sophorolipids as stable and non-toxic surface coatings for IONP-based
24
25
26
27
28 biomedical and biotechnological applications.
29
30
31
32
33
34
35
36
37

38 Introduction

39
40

41 Superparamagnetic iron oxide nanoparticles are used in applications such as drug
42
43
44 delivery, MRI or hyperthermia, for which iron oxide cores smaller than ~20 nm in diameter
45
46
47 are coated with weakly adsorbed polymers (often dextran¹⁻³) or lipids.⁴ However,
48
49
50
51 physisorbed shells lead to low dispersant densities on the particle surface and low
52
53
54
55 colloidal stability over time. Controlled grafting of chemically linked dispersants yields
56
57
58
59
60

denser and more stable shells; therefore, such approaches have received increasing attention.⁵⁻⁶ With improvements in the synthesis, monodisperse (SD < 5 %) iron oxide nanoparticles have been developed,⁷ but their hydrophobic (mostly oleic acid) shell of weakly adhering ligands is not compatible with biomedical applications. Consequently, the original ligands must be replaced by chemisorbed ligands that provide a water-soluble and sterically stabilizing shell, crucial for colloidal stability in biological fluids. Ligand exchange protocols for highly hydrated and chemically grafted ligands, mainly based on poly(ethylene glycol) (PEG), have therefore been developed.⁸ PEG provides excellent colloidal stability and long circulation times of the particles in the body due to limited opsonization (protein binding and elimination of the particles by the reticuloendothelial system),⁹ but PEG does not provide targeting specificity and has increasingly been claimed to be subject to antibody production.¹⁰ Coating of nanoparticles with biological or biomimetic ligands could address both these drawbacks; to this end, other surface ligands are tested, including carbohydrates.

Carbohydrate-grafted nanoparticles are generally referred to as glyconanoparticles¹¹⁻¹² and they are constituted by a nanoparticle core (e.g. gold or iron oxide) and a shell

composed of a glycoconjugate, a term which refers to a molecule composed of a carbohydrate on one side and a binding group on the other. Since specific carbohydrates are known to selectively bind proteins,¹³⁻¹⁵ glyconanoparticles generally contain mannose, galactose, fucose or sialic acid, which are well-known saccharides targeting carbohydrate receptors on tumor cells.^{14, 16} The binding group must be selected with the type of core particle surface in mind. Typically, thiols are used to graft to gold nanoparticles while silanes are used to graft to oxide nanoparticles.^{12, 16-17} The main drawback of these fully synthetic systems is the complex synthesis of the glycoconjugate ligands, mainly due to the classically tedious sugar chemistry, and for this reason alternative routes to prepare glyconanoparticles are worth exploring.

In the past fifteen years, a number of research groups has shown the potential of using natural capping agents for metallic nanoparticles.¹⁸⁻¹⁹ However, the field is still in its infancy due to the poor control over both particle size distribution and composition of the shell. A promising group of novel capping agents are glycoconjugates obtained through microbial synthesis. This class of molecules, in particular sophorolipids (SL), is the most studied in the field of nanoparticle stabilization. SL are bolaform, non-toxic, bio-based

glycolipids produced by the yeast *Starmerella bombicola* and characterized by a bulky sophorose headgroup and a free carboxyl group.²⁰ SL are known to have natural targeting abilities, anti-cancer²¹ and anti-bacterial²² properties. SL were previously used to stabilize metal nanoparticles (Ag, Co)²³⁻²⁵ and iron oxide nanoparticles (IONP).²⁶ The interaction between SL and the nanoparticles was attributed to the complexing of the accessible COO⁻ group to the surface; however, low grafting densities, poor control over polydispersity, shell composition and even nanoparticle structure show that further enhancement of SL-coated iron oxide nanoparticle synthesis and structure are required for applications. Here, we propose a new approach based on the use of size-controlled monodisperse IONP and SL having the COOH group modified with nitrodopamine (NDA) via an amide bond. NDA is an anchor molecule that has been used to bind dispersants strongly to IONP.²⁷⁻²⁸ We demonstrate grafting of a monolayer SL shell by a combination of SANS and synchrotron SAXS. Furthermore, we show the resulting excellent colloidal stability of the SL-IONP in physiological buffers and protein concentrations, paired with absent cytotoxicity. This work opens opportunities for the application of glycosylated IONP obtained from natural glycoconjugates, combining strong colloidal stability and

1
2
3 water solubility with the imaging, tracking and therapeutic abilities contributed by the
4
5
6
7 IONP core.
8
9
10
11

12 Experimental Section

13
14
15
16 **Materials.** All Chemicals were purchased from Sigma-Aldrich and all solvents from
17
18
19 Roth. Chemicals were used as received without further purification (see supporting
20
21
22 information for details). Sophorolipids (Sopholiance, batch number: 11103A, dry content:
23
24
25
26 $60 \pm 6 \%$) are purchased from Soliance, France.
27
28
29

30 **Synthesis of sophorolipids (SL).** Acidic sophorolipids have been prepared from a
31
32
33 commercial batch of a sophorolipid mixture using alkaline hydrolysis to convert the crude
34
35
36 lactonic/acidic mixture into a fully acidic sophorolipid compound. Extraction and
37
38
39 purification have been performed using the method N°2 described earlier,²⁹ to which one
40
41
42 should refer for a typical ¹H solution NMR fingerprint. The compound used here is majorly
43
44
45
46
47
48 (> 80%) composed of the acidic subterminal C18:1-*cis* form that forms micelles.³⁰
49
50

51 **Synthesis of nitrodopamide-SL (SL-NDA) ligands.** 6-Nitrodopamine-hemisulfate (NDA-
52
53
54 HSO₄) was synthesized according to literature with slight modifications.³¹ SL-NDA was
55
56
57
58
59
60

synthesized by (1-cyano-2-ethoxy-2-oxoethylidenaminoxy)dimethylamino-morpholino-carbenium hexafluorophosphate (COMU) mediated peptide-coupling reactions.³²⁻³³

Briefly, 100 mg (0.16 mmol) of SL were pre-activated with 1.5 eq COMU and 1 eq *N*-methylmorpholine in 7 mL dimethylformamide (DMF) at RT for 10 minutes. The mixture was cooled to 0 °C and a mix of 1eq NDA and 1eq *N*-methylmorpholine in 5 mL DMF was slowly dropped to the activated acid. The mixture reacted for 1 h at 0 °C and subsequently 3 h at RT. The solvent was evaporated and 4 mL of Milli-Q were added to the crude product. The product was transferred into butanol (BuOH) and extracted thrice with Milli-Q, once with 1.5 M HCl and finally washed with pure Milli-Q. BuOH was evaporated and the product was vacuum-dried. The coupling of SL to NDA was confirmed by FTIR and by solution ¹H NMR (see Figure S1 for details), showing the coexisting 1:1 ratio between SL and NDA together with the downfield shift, from 3.05 ppm to 3.47 ppm, attributed to the α-CH₂ in NDA and respectively in an amine and amide environment.^{32, 34} One can also observe the parallel upfield shift, from 2.30 ppm to 2.18 ppm of α-CH₂ in SL for respectively an amine and an amide environment. NMR excludes the presence of unreacted free NDA, but we must acknowledge the presence of residual COMU and

1
2
3 unreacted SL (between 10 and 20 mol% with respect to SL-NDA), which due to similar
4
5
6
7 physico-chemical properties were not removed by washing the sample. However, COMU
8
9
10 and unreacted SL do not have the affinity to the iron oxide nanoparticle surface to displace
11
12
13
14 oleic acid or interfere with the ligand displacement by SL-NDA.
15
16

17 **Synthesis of iron oxide nanoparticles (IONP).** Iron oxide NP (3-10 nm in diameter)
18
19
20 coated with oleic acid (OA) were synthesized by thermal decomposition of iron
21
22
23 pentacarbonyl according to a heat-up procedure originally proposed by Hyeon *et al.*³⁵ that
24
25
26
27 was slightly modified as described in ⁸. Details for the molar ratios of reactants can be
28
29
30
31 found in the supporting information (Table S1). Larger, 14.1 nm IONP were synthesized
32
33
34 via a modified two-step method introduced first by Park *et al.*³⁶ and previously by us.⁹
35
36
37
38 IONP cores with diameters of 3.1, 4.6 and 14.1 nm were used for functionalization with
39
40
41
42 sophorolipids and further experiments.
43
44

45 **Ligand exchange and purification.** SL-IONP were synthesized according to a slightly
46
47
48 modified method published earlier.⁸ For 4.6 nm cores, 0.9 g of as-synthesized IONP
49
50
51 coated with oleic acid were dispersed in 20 mL DMF. 0.183 g of SL-NDA were added and
52
53
54
55 the mixture was sonicated for 28 h at slightly elevated temperature. Amounts of ligand
56
57
58
59
60

1
2
3 were adjusted for the smaller 3.1 nm IONP to account for the difference in surface area
4
5
6
7 and the same surface area to ligand ratio was used. However, for the 14.1 nm cores we
8
9
10 used a much larger excess of ligand (10x with respect to surface area), supposing that a
11
12
13 larger excess of ligand will aid in achieving a high surface coverage. After ligand
14
15
16
17 exchange, the SL-IONP were washed three times with n-hexane to remove released OA.
18
19
20
21 In a final step, the solvent was evaporated and the IONP were lyophilized. The lyophilized
22
23
24 SL-IONP were redispersed in Milli-Q and dialysed in a 12-14 kDa MWCO dialysis bag
25
26
27
28 against 5 L Milli-Q to remove excess SL-NDA ligands. Alternatively, SL-IONP were
29
30
31 purified by repeated (5x) membrane centrifugation in 50 kDa MWCO Amicon filter units.
32
33
34
35 The purified SL-IONP were lyophilized and could be stored for more than two years as a
36
37
38 dark-brown powder. The yield was 96 % calculated with respect to the initial amount of
39
40
41 IONP cores. SL-IONP were analyzed by TEM, TGA, ATR-FTIR and DLS.
42
43
44

45 The samples were named according to their core diameter measured by TEM: 3.1 nm
46
47
48 SL-IONP (diameter 3.1 nm), 4.6 nm SL-IONP (diameter 4.6 nm) and 14.1 nm SL-IONP
49
50
51 (diameter 14.1 nm)
52
53
54

55 **Cell growth.** U937 cells were grown under conditions reported in ⁹.
56
57
58
59
60

Toxicity test. Toxicity measurements were performed with the PrestoBlue® Cell Viability assay. Briefly, 2×10^4 cells were seeded on 96-well plates using 200 μ l medium per well and incubated for 16 h (each 6 repeats for sample and control). 5 μ l nanoparticle dispersion (1 mg Fe/ml) were added and incubated for either 2 or 20 h. As control 5 μ l Milli-Q water were added. After incubation, 20 μ l PrestoBlue® were added and plates were incubated for one hour. A Tecan infinite F200 plate reader with excitation filter 560 ± 20 nm and emission filter 595 ± 35 nm was used to read the plates. The background was determined by measuring medium with presto blue at 10 % (v/v).

Viability was calculated as:

$$\frac{X}{X_c} * 100 \% \quad (1)$$

with X the average value determined for cells incubated with SL-IONP and X_c the average value measured for control cells.

SL-IONP uptake by cells and iron quantification. For uptake experiments, 2×10^6 cells (monocytes) and 1×10^6 cells (MCF-7) were seeded in a total volume of 5 ml in 6-well plates and incubated for 12 h. 50 μ L of a 1 mg/mL Fe dispersion of SL-IONP were added

1
2
3 and the cells were incubated for 20 hours. The control well was incubated with 50 μ L Milli-
4
5
6
7 Q water. After incubation, cells were centrifuged and consequently washed three times
8
9
10 with PBS to remove IONP that were not taken up the cells. Iron quantification was done
11
12
13 by a modified Ferrozine test as described previously.⁹ For each type of SL-IONP at least
14
15
16
17 5 wells were prepared in parallel and 3 independent repetitions were performed, resulting
18
19
20
21 in a minimum of 9 data points for each type of SL-IONP.
22
23

24 **Protein quantification.** The Rotitm-NanoQuant protein detection assay was used for
25
26
27 protein quantification according to the manufacturer's protocol. BSA dissolved in 50 mM
28
29
30 NaOH was used as a standard.
31
32
33

34 **Transmission electron microscopy (TEM) and analysis.** TEM images were recorded on
35
36
37 a FEI Tecnai G2 20 transmission electron microscope at 160 kV for low resolution and
38
39
40 200 kV for high resolution imaging. Samples were drop-casted from toluene dispersions
41
42
43 (IONP capped by oleic acid) or aqueous dispersions (SL-IONP) onto a 300-mesh carbon-
44
45
46 coated copper. The Pebbles³⁷ software package was used to determine the size
47
48
49 distributions from the micrographs. Approximately 900 IONP were used for size
50
51
52
53 determination by Pebbles for each sample of nanoparticles.
54
55
56
57
58
59
60

Transmission Electron Microscopy experiments under cryogenic conditions (cryo-TEM). Cryo-TEM images were recorded on a FEI Tecnai 120 twin microscope at 120 kV with a Gatan Orius CCD numeric camera and a Gatan Cryoholder (Gatan 626DH, Gatan). DigitalMicrograph™ software was used for image acquisition. A custom-built device was used for cryo-fixation. SL-IONP aqueous dispersions and SL-NDA solutions were deposited on glow-discharged holey carbon copper grids (Quantifoil R2/2, Germany). The grids were immediately plunge-freezed by dipping into liquid ethane at -180 °C and transferred into liquid nitrogen. All grids were stored in liquid nitrogen.

Thermogravimetric Analysis (TGA). TGA data were measured using a Mettler-Toledo TGA/DSC 1 STAR System in a temperature range of 25-650 °C with 10 K/min. A stream of 80 mL/s of synthetic air was used to ensure complete combustion of the SL-NDA ligands since NDA is known to polymerize by pyrolysis under N₂. 0.5 - 1.5 mg sample were measured, The total organic content (TOC) was calculated as the fraction of mass loss up to 650 °C.

Dynamic Light Scattering (DLS). DLS was measured on a Malvern Zetasizer Nano-ZS at 20 °C. Instrument settings: Fe₃O₄, Milli-Q as dispersant (0.89 mPa·s, RI=1.33), 3

individual measurements were performed with 13 runs of 10 s each. All samples of SL-IONP were measured at a concentration of 0.5 mg_{Fe}/mL. The nanoparticles were dispersed in water (2 mg/mL) and 10 % fetal calf serum for experiments in which the temperature was varied from 20 °C to 70 °C and back with 5 °C steps and an equilibration time of 2 minutes. At each temperature, 3 individual measurements with 13 runs of 10 s were recorded.

¹H Nuclear magnetic resonance (NMR) measurements. ¹H NMR data were acquired on a Bruker Avance III 300 spectrometer using a 5 mm ¹H-X BBFO probe, 16 transients with 1 s recycling delay. The value chemical shifts (δ) are reported in ppm at 298 K using tetramethylsilane (TMS) as reference. MeOD-d₄ was used as solvent. COSY spectra were acquired using a standard pulse program from the Avance III library, with 1 s recycling delay, 8 transients per scan and 256 spectra acquired in the F1 dimension.

Attenuated total reflection – Fourier transform infra-red (ATR-FTIR) measurements. IR spectra of the lyophilized samples were recorded on a Perkin Elmer 400 FTIR

spectrometer with diamond single-reflection ATR equipment and a resolution of 4 cm^{-1} , averaging 32 scans.

Small Angle X-ray Scattering (SAXS). SAXS patterns were measured using a Dectris-Pilatus 1M detector with a resolution of 981×1043 pixels and a pixel size of $172 \times 172\text{ }\mu\text{m}$ at the Dutch-Belgian Beamline (DUBBLE) station BM26B of the European Synchrotron Radiation Facility (ESRF) in Grenoble, France. The sample to SAXS detector distance was set to ca. 3100 mm using a wavelength of $1.033\text{ }\text{\AA}$. SAXS patterns were integrated azimuthally to obtain a typical $I(q)$ spectrum. A quartz capillary, used as sample holder, was filled with Milli-Q grade water, used as solvent, and then with the sample dispersion. To eliminate errors in the background subtraction process due to inhomogeneities of the capillary walls, great care was taken to analyze both the water and sample solutions at the same position. Solvent contribution was subtracted during the data treatment. Data were corrected for the transmission of the direct beam and presented in absolute scale. The q -range calibration was made using a silver behenate standard sample ($d_{ref} = 58.38\text{ }\text{\AA}$)

Small Angle Neutron Scattering (SANS). SANS was performed at the Institut Laue-Langevin (ILL, Grenoble, France) on D16 and D33 beamlines. The spectrometer configurations were adjusted as follows. *D16 beamline* ($3.5 \cdot 10^{-2} \text{ \AA}^{-1} < q < 0.5 \text{ \AA}^{-1}$): neutron wavelength, $\lambda = 4.5 \text{ \AA}$, sample-to-detector distance = 955 mm, sample and detector rotation angles: $\omega = 5.5^\circ$ and $2\theta = 11^\circ$, acquisition time = 60 min. *D33 beamline* ($5.0 \cdot 10^{-3} \text{ \AA}^{-1} < q < 0.3 \text{ \AA}^{-1}$): neutron wavelengths: $\lambda_1 = 4.6 \text{ \AA}$ and $\lambda_2 = 13.0 \text{ \AA}$ sample-to-detector distances = 2 m, 10 m, acquisition time = 10 min (in D₂O), 45 min (in MeOD-d4). For both spectrometers: neutron absorber: Boron Carbide (B₄C), thickness of quartz Hellma cells: 1 mm, temperature (controlled): $T = 25^\circ\text{C}$, background: 99.9 % D₂O. Direct beam, empty cell and H₂O were also recorded for both spectrometers. The background sample (D₂O) signal was subtracted from the experimental data. q is defined as $(4\pi/\lambda)\sin\vartheta/2$ where ϑ is the scattering angle between the incident and the scattered neutron beams. Data treatment was done with the home-made software package provided at the beamline. Direct determination of the number of neutrons in the incident beam and the detector cell solid angle allowed obtaining absolute values of the scattering intensity. The acquired 2-D raw data were corrected for the scattering of the ambient background and the empty

cell. Further, the data were normalized by the neutron flux on the samples to yield an absolute scale (cross section per unit volume). The data were radially averaged to yield the 1-D intensity distribution $I(q)$.

Fit of small angle scattering data (SAXS, SANS). All data presented were fitted using a classical core-shell sphere form factor model (assuming a unitary structure factor in the analyzed range of q -values),³⁸ using the SasView 3.1.2 software (CoreShellModel) available free of charge on the developers' website.³⁹ In the model, we consider the core to be composed of iron oxide and the shell of a homogeneous SL-NDA layer of variable thickness. One should be aware that this is a strong approximation, because SL-NDA could be described by at least three different regions of inequivalent densities: sophorose, oleic acid and NDA. Nonetheless, the goal of the fitting analysis in this work is not to precisely describe the morphology of the of the nanoparticle shell, but to demonstrate that SL-IONP have a shell with a thickness compatible with a monolayer of SL-NDA; the homogeneous core-shell model provides an adequate model with a minimum number of fitting variables to determine the presence and thickness of a reasonably homogeneous shell. All theoretical and technical details of both the model and fitting function can be

found at on the developers' homepage.³⁹ The following parameters (Table 1) characterize the fitting function:

Table 1. Structural parameters used in the core-shell sphere model to fit both the SANS and SAXS data recorded for the SL-IONP.

Parameter	Description	Fixed	Variable
d	Core diameter	x	
t	Shell Thickness		x
ρ_c	Scattering Length Density (SLD) of the IONP core	x	
ρ_s	Scattering Length Density (SLD) of the SL shell		x
ρ_{solv}	Scattering Length Density (SLD) of the solvent (H ₂ O and D ₂ O for X-ray and neutron scattering experiments respectively)	x	
scale	Volume fraction	x	
background	Background level		x
PDI	Polydispersity		x

The fixed parameters of the fitting function vary according to the type of technique used to study the samples. The fixed values which we have used in this work are reported

hereafter. The nanoparticle diameter d is determined independently by TEM and SAXS.

The SLD values have been determined using the SLD calculator tool in the SasView software. $\rho_{solv}(\text{X-ray}) = 9.410 \times 10^{-6} \text{ \AA}^{-2}$ and $\rho_{solv}(\text{neutron}) = 6.4 \times 10^{-6} \text{ \AA}^{-2}$, where $\rho_{solv}(\text{X-ray})$ and $\rho_{solv}(\text{neutron})$ respectively refer to the X-ray and neutron SLD of H_2O and D_2O . $\rho_c(\text{X-ray}) = 40.0 \times 10^{-6} \text{ \AA}^{-2}$ and $\rho_c(\text{neutron}) = 6.9 \times 10^{-6} \text{ \AA}^{-2}$ refer to the X-ray and neutron SLD of iron oxide respectively, for which we have used the bulk density value of 5.17 g.cm^{-3} . Polydispersity was allowed to vary between 0.1 and 0.15 and the volume fraction was set to 0.4 % for samples 3.1 nm SL-IONP and 4.6 nm SL-IONP and to 0.1 % for sample 14.1 nm SL-IONP, the smaller value coming from the larger amount of SL-NDA in the 14.1 nm SL-IONP sample (please refer to main text for more details). In the final fitting procedure, t and ρ_s are the only structural variable parameters, while the background level is set as variable mainly to adjust the quality of the final fit. The typical size and composition of SL-NDA also imposes several constraints on the effective t and ρ_s values. One can reasonably consider $2.0 < t \text{ (nm)} < 3.5$ for a single SL-NDA layer, where the lower and upper limits consider plausible molecular bending and stretching; meanwhile, $9.6 < \rho_s(\text{X-ray}) (10^{-6} \text{ \AA}^{-2}) < 12$ and $1 < \rho_s(\text{neutron}) (10^{-6} \text{ \AA}^{-2}) < 5$ reasonable

identify the range for both the X-ray and neutron shell SLD values, which can vary between a highly hydrated shell and a poorly hydrated organic layer.

Results and Discussion

IONP synthesis, ligand exchange and purification. Spherical, monodisperse and single-crystalline iron oxide nanoparticles with diameters of 3.1 ± 0.3 nm ($\sigma = 8.0$ %), 4.6 ± 0.3 nm ($\sigma = 7.0$ %) and 14.1 ± 0.9 nm ($\sigma = 6.9$ %) were synthesized and characterized by HR- and LR-TEM. Figure S2 shows TEM micrographs of as-synthesized monodisperse oleic acid-coated iron oxide cores.

The exchange of hydrophobic oleic acid for sophorolipid-nitrodopamide ligand was performed using a method established earlier⁸ and yielded well-dispersed SL-NDA coated IONP (SL-IONP) with three different core sizes (Figure 1). Single-crystallinity of the IONP cores was revealed by HR-TEM (inset in Figure 1C and Figure S3) and the lattice spacing in the (220) direction was measured to be 0.29 nm. The electron diffraction pattern (inset Figure 1B) shows the high crystallinity of the IONP. The determined ratios of d -spacings show good agreement with the JCPDS database values given for

maghemite or magnetite.⁴⁰ Single-crystalline iron oxide nanoparticles (magnetite and maghemite) in this size-range are known to be superparamagnetic, thereby possessing attractive properties for biomedical imaging and drug delivery.

Figure 1. TEM micrographs of three sizes of SL-IONP of sizes A) 3.1 ± 0.3 nm, B) 4.6 ± 0.3 nm and C) 14.1 ± 0.9 nm. The insets in B) and C) show the electron ring diffraction pattern and a high-resolution micrograph of 14.1 nm IONP, revealing the high crystallinity of the single crystal cores.

To demonstrate colloidal stability of SL-grafted IONP, excess ligand should be removed from the nanoparticle dispersion. Thus, SL-IONP were purified using two common

1
2
3
4 purification methods for nanoparticles, namely dialysis and membrane centrifugation to
5
6
7 remove the excess of the micelle-forming glycolipid ligand.
8
9

10 The functionalization of the IONP with SL-NDA through ligand replacement was first
11
12 evaluated using ATR-FTIR. Figure 2 shows the FTIR spectra of the compounds and
13
14 products. NDA has a characteristic C-O stretch band at 1272 cm^{-1} ,⁴¹ which can be found
15
16
17 in the product of the purified SL-NDA but not in pure SL. Likewise, the “sugar bands”
18
19
20 corresponding to C-O and C-O-C stretch modes at 1021 cm^{-1} and 1069 cm^{-1} ,⁴²
21
22
23 respectively, from the sophorolipid are also strong in the SL-NDA product. Finally, the
24
25
26 bands of the amide bond formed between SL and NDA are clearly visible in SL-NDA.
27
28
29 Figure 2 shows that two characteristic bands of oleic acid, the C=O stretch at 1707 cm^{-1}
30
31
32 (which is slightly higher than for sophorolipid) and the out-of-plane O-H stretch at 963
33
34
35 cm^{-1} are absent or insignificant compared to the characteristic bands of the sophorolipid
36
37
38 and NDA.³² In summary, the ATR-FTIR results strongly support successful ligand
39
40
41 replacement of the oleic acid by SL-NDA to form iron oxide nanoparticles with a shell of
42
43
44 sophorolipid, strongly linked by nitrodopamide.
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Figure 2 ATR-FTIR spectra of as-synthesized IONP (oleic acid capped, green), nitrodopamine (grey), sophorolipid-NDA (blue), SL-IONP 4.6 nm (red) and sophorolipid (black). Curves have been shifted vertically for clarity. With attributions: $\delta_{(C=O)-OH}$ (oleic acid): 963 cm^{-1} , ν_{C-O} (sugar): 1021 and 1069 cm^{-1} , ν_{C-O} (catechol): 1272 cm^{-1} , $\nu_s(\text{NO}_2)$: 1331 cm^{-1} , ν_{C-C} (catechol): 1507 cm^{-1} , $\nu_a(\text{NO}_2)$: 1538 cm^{-1} , $\nu_{C=O}$ (amide): 1643 cm^{-1} , $\nu_{C=O}$ (amide) and δ_{N-H} (amide): 1538 cm^{-1} and , $\nu_{C=O}$ (carboxylic): 1707 cm^{-1} .⁴¹⁻⁴³

The average grafting density of SL-NDA ligand irreversibly bound to the Fe-ions at the IONP surface was evaluated after purification by TGA. Typical TGA weight loss profiles for 3.1 nm SL-IONP, 4.6 nm SL-IONP and 14.1 nm SL-IONP are shown in Figure S4. The total organic content (TOC) was determined as the fraction of mass loss up to 650 °C. If all excess ligand is removed, the TOC can be converted into the dispersant grafting density using the known molecular weight of SL-NDA, the defined average iron oxide core area determined by TEM and a known iron oxide core density of 5.17 g/cm³. We thus assume for this calculation that all oleic acid was replaced by SL-NDA during the rigorous ligand replacement and purification. Earlier studies have shown that ligand replacement of OA by hydrophobic ligands is challenging and can leave significant residues within the nanoparticle shell.^{32, 44} If this is the case, the amount of SL-NDA grafted to the IONP would be overestimated. However, we have previously investigated the ligand replacement and purification protocol used here and shown that the amount of residual OA is below the detection limit by ATR-FTIR. As discussed above, the ATR-FTIR data shown in Figure 2 supports this finding. Additionally, the calculation of the grafting density based on the TGA data can yield a higher value than the actual grafting density if there

is also free ligand present in a sample. We therefore list the average number of ligands ($n_{ligands}$) per nm² in Table 2 calculated in the same way for differently sized IONP purified by dialysis or membrane centrifugation (Amicon 50 kDa MWCO) to take into account the possible presence of free ligand.

Table 2. TGA results for SL-IONP of three different core sizes. Total organic contents (TOC) was converted into average number of ligands/nm² ($n_{ligands}/nm^2$) using the known SL-NDA Mw (902 g/mol).

SL-IONP type	$n_{ligands}/nm^2$ [dispersant/nm ²]	TOC [%w/w]
3.1 ± 0.3 nm dialyzed	8.0 ^a	82.3 ^a
3.1 ± 0.3 nm Amicon	7.1 ^a	80.5 ^a
4.6 ± 0.3 nm dialyzed	4.3	60.8
4.6 ± 0.3 nm Amicon	3.3	55.6
14.1 ± 0.9 nm dialyzed	37.4 ^a	82.1 ^a

1
2
3
4 14.1 ± 0.9 nm

5 45.2^a

6 84.8^a

7 Amicon

8 ^a Samples contained large amounts of excess ligand.
9
10
11
12
13

14 The limiting factor for ligand adsorption to the IONP surface is given by the number of
15
16
17 surface iron sites (3.5 - 5.9 depending on crystal plane)⁴⁵ or the spatial requirements
18
19
20 (footprint) of the ligand. The hydrated sugar headgroup and the unsaturated fatty acid
21
22
23 structure of SL could sterically hinder achieving the maximum grafting density of NDA,
24
25
26 but this steric blocking is reduced by the high curvature of the IONP that provides much
27
28
29 larger volume for the headgroup than for the binding group. The grafting density of the
30
31
32 deacetylated acidic form of sophorolipids (the same used in this work) varies according
33
34
35 to the system under study; one finds between 2 and 3 molecules/nm² on a flat gold
36
37
38 surface (evaluated by X-ray photoelectron spectroscopy and quartz crystal microbalance
39
40
41 with dissipation monitoring),⁴⁶ 0.96 molecules/nm² at the air/water interface (surface
42
43
44 tension)⁴⁷ and between 0.5 and 1.0 molecules/nm² in micelles respectively at the
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

is within the range of plausible grafting densities taking both the density of grafting sites and steric blocking during grafting into account. The other systems display values of SL-NDA/nm² that are higher than the number of possible grafting sites on the IONP surface, which sets an absolute upper bound on the grafting density. Hence, the observed values above 5 molecules/nm² (Table 2) must be due to excess ligand that is not grafted to the IONP surface. The excess ligand could not be removed with Amicon filters and dialysis could also not reduce the organic content of the samples. It has been reported that sophorolipids form micellar structures in water,²⁰ and cryo-TEM micrographs in Figure 3A and Figure S5 demonstrate that also SL-NDA self-assembles into both spherical and filamentous structures. Since it is not a goal of this work to describe the self-assembly properties of SL-NDA in water, we prefer not to go into descriptive details; we will simply refer to a general micellar and filamentous self-assembled phase of SL-NDA in coexistence with SL-IONP.

Figures 3B-C and Figures S6 and S7 show cryo-TEM images of 14.1 and 4.6 nm SL-IONP purified by membrane centrifugation. The 14.1 nm cores have the highest organic content according to TGA, while the 4.6 nm cores have an organic content compatible

with maximum grafting of SL-NDA to the IONP surface. In addition to the nicely dispersed SL-IONP with high electron density contrast, large and elongated structures with lower contrast are present primarily in the 14.1 nm core sample; these strongly resemble the filamentous structures formed by SL-NDA in water, depicted in Figure 3A.

Figure 3. Cryo-TEM micrographs in H₂O of A) SL-NDA, B) 14.1 nm SL-IONP and C) 4.6 nm SL-IONP. The blue arrows indicate lamellar structures formed by excess ligand; the black dots are nanoparticles (see Figure S6 and S7 for enlarged pictures).

This feature suggests why SL-NDA could not be fully removed by dialysis or by membrane centrifugation: the cut-off pore size of the membranes is larger than a single SL-NDA molecule, but smaller than their self-assembled phase. Dialysis should in

principle allow for removal of excess lipid from the sample, but in practice, self-aggregating molecules with a low critical aggregation constant or very slow kinetics of desorption (low k_{off}) will make dialysis very inefficient; this is apparently the case for dialysis of SL-NDA forming large and stable structures. An attempt to improve the dialysis with salt (NaCl) by shifting the critical micelle concentration failed for this system since SL-NDA micelles are insensitive to salt (*cf.* SANS data below). Interestingly, the small core sample (4.6 nm) had much less excess ligand after purification than the large core sample. This could tentatively be explained by that for the larger cores a larger excess of SL-NDA to IONP surface area was used, which favors growth of more and larger micelles during grafting or during resuspension. The 4.6 nm SL-grafted IONP presumably had a lower amount of excess SL-NDA in solution after grafting and consequently a negligible excess of SL-NDA in the dispersion after purification. This interpretation of the purification and TGA data is supported by cryo-TEM (Figure 3C and Figure S7), in which many stabilized nanoparticles are observed, but only a limited amount of SL-NDA. With the difficulty to purify nanoparticles from SL-NDA micelles and filaments, one can try to minimize the excess of SL-NDA during grafting to suppress micelle formation. However,

1
2
3 this easily leads to inefficient grafting and sub-monolayer dispersant grafting density on
4
5
6
7 the particle surface. Grafting with large SL-NDA excess is therefore recommended for
8
9
10 applications where excess micelles can be tolerated but particle colloidal stability is of
11
12
13
14 paramount importance.
15
16
17
18
19
20

21 **Characterization of the SL-NDA ligand shell with SANS and SAXS.** It is typically
22
23
24 assumed that the high colloidal stability of coated nanoparticles is explained by the
25
26
27 presence of a single layer of ligand; this argument may also hold for the SL-IONP
28
29
30 presented here. However, most classical techniques, like DLS or TEM, on which this
31
32
33 assumption is based, are generally not providing *in situ* or detailed enough data to
34
35
36 quantitatively investigate the presence, structure and properties of the shell in support of
37
38
39 this hypothesis. SAXS, which is sometimes used for this purpose, is mostly not suitable
40
41
42 for hybrid inorganic-organic nanoparticles, because the contrast between the solvent and
43
44
45 the electron-rich inorganic core generally masks the signal of the shell resulting from the
46
47
48 poor contrast between the solvent and the shell. The combined use of X-ray and neutron
49
50
51 scattering is the only method to probe the structural characteristics such as the thickness
52
53
54
55
56
57
58
59
60

of the nanoparticle organic shell. In fact, neutrons, being scattered by the atom nuclei, are less sensitive to atoms with high atomic number. We therefore combined SAXS and SANS as a reliable method to estimate the extension of the SL-NDA shell for SL-IONP purified by Amicon filtration, that had a low excess of ligand in micelles.

SAXS was used to determine and confirm the size of the IONP cores. Under X-ray irradiation, the inorganic core of SL-IONP nanoparticles has a very high contrast ($\rho_{\text{soln}} - \rho_c = -30.60 \times 10^{-6} \text{ \AA}^{-2}$, Table 3) if compared to the shell-solvent ($\rho_{\text{soln}} - \rho_s = -0.57 \times 10^{-6} \text{ \AA}^{-2}$, Table 3); for this reason, SAXS only provides structural information on the nanoparticle cores. Figure 4A (green circles) shows the SAXS profile for 4.6 nm SL-IONP. The best fit using a core-shell sphere form factor can be obtained using an average nanoparticle core diameter of $4.8 \pm 0.4 \text{ nm}$ and a null value for the shell thickness. This is in very good agreement with the diameter obtained by TEM ($4.6 \pm 0.3 \text{ nm}$) within the experimental errors. The corresponding SANS pattern (Figure 4A, blue circles) is shifted towards the low- q region, which signifies sensitivity to the larger size resulting from the much higher contrast of neutrons to the lipid shell.

The SANS pattern can be modeled using an imposed (from SAXS data) core diameter of $d = 4.8 \pm 0.4$ nm and a shell thickness of $t = 2.5 \pm 0.2$ nm obtained by fitting a core-shell model to the scattering data. This simple model assumes a uniform scattering length density throughout the shell, which is clearly a simplification, but it can be used to estimate the shell thickness by fitting the shift of the first minimum of the form factor. The calculated length of SL, considering the 120° angle due to the mono-unsaturation, can be estimated to 2.6 nm, where 1.6 nm are attributed to the fatty acid⁴⁸ and 1 nm to sophorose, in analogy to the size of maltose, a common disaccharide.⁴⁹ The size of NDA is estimated to about 1 nm.⁵⁰ Under these conditions, the maximum expected size for SL-NDA is about 3.6 nm. The modeled thickness of $t = 2.5 \pm 0.2$ nm is shorter than the maximum monolayer thickness and demonstrates that the shell of monodisperse SL-IONP is comprised of a single layer of SL-NDA of less than maximum density. From the fit, one can also evaluate an average shell scattering length density of $\rho_s = 3.39 \times 10^{-6} \text{ } 10^{-6} \text{ } \text{\AA}^{-2}$, a value which is reasonable for a hydrated (in D_2O) organic lipid layer.⁵¹ The lower density or slightly lower thickness that is observed can be explained in several ways. One can, e.g., consider that the shell could contain other smaller ligands than SL-NDA, such as

1
2
3 residual catalyst (COMU), oleic acid or unreacted NDA or SL. The combination of TGA,
4
5
6
7 FTIR and NMR data make this hypothesis unlikely. COMU, which was observed as
8
9
10 residue in the SL-NDA batches used for grafting the NPs, has very low affinity for the iron
11
12
13 oxide surface. All techniques employed show no evidence of residual OA, while ^1H NMR
14
15
16 shows that the SL-NDA does not contain free NDA. A residual amount of unreacted SL
17
18
19 (10-20 mol% with respect to SL-NDA) is present. The carboxyl endgroup of SL has high
20
21
22 affinity to the IONP, but NDA functionalized ligands possess much higher affinity and
23
24
25 outcompete carboxyl anchored ligands on the surface. This is demonstrated by FTIR
26
27
28 showing the successful ligand replacement of OA by SL-NDA and previous studies of e.g.
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

OA with SL failed to produce water-soluble particles, strongly suggesting that replacement of OA will be driven by SL-NDA. Thus, although our data do not allow its exclusion, we do not expect significant amounts of unmodified SL on the IONP.

A more plausible explanation for the thin shell is that the SL-NDA is grafted on a highly curved surface, which provides a rapidly increasing conical volume to the SL-NDA even at the highest grafting density.⁵² This allows for a large tilt or bending of the molecules,

1
2
3 i.e. a lower effective shell thickness. The extended length of SL is calculated according
4
5
6
7 to the classical Tanford formula. However, SL is known to adopt a bent configuration,
8
9
10 which yields an effectively thinner layer than theoretically calculated. For instance, XPS
11
12
13 has shown that a single self-assembled monolayer of cysteamine-modified SL grafted
14
15
16 onto flat gold has a thickness of only 3.0 ± 0.2 nm,⁴² while small-angle X-ray and neutron
17
18
19 scattering measurements showed that the total radius of an acidic SL micelle is generally
20
21
22 below 4 nm, i.e, approximately the size of a single SL molecule.⁵³⁻⁵⁴ A layer grafted on a
23
24
25
26
27 highly curved NP would be expected to yield a thickness in between these estimates.
28
29
30

31 Finally, one should not exclude that the simplification of assuming a uniform scattering
32
33
34 length density, which could be invalidated by the conical ligand volume and the highly
35
36
37 hydrated headgroup, would also lead to a fit predicting an effectively thinner shell.
38
39
40
41
42 Whichever the dominant of the two latter explanations, the SAXS/SANS data demonstrate
43
44
45 a single thin SL-NDA layer serving as the stabilizing shell on the IONP.
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Figure 4. A) SANS (blue) and SAXS (green) profiles for 4.6 nm SL-IONP in D_2O . The red lines show the fit obtained from a core-shell sphere model. The best fit for SAXS was obtained using a core diameter of 4.8 nm and a null value for the shell. B) Comparison of the SANS data of 4.6 nm (blue) and 3.1 nm SL-IONP (grey). As expected, the profile for smaller SL-IONP is shifted to larger q . The red lines show the fit obtained with a homogeneous core-shell model.

Similar results are obtained for the smaller 3.1 nm SL-IONP (Figure 4B). The SANS pattern can be modelled with a fixed (from SAXS data) core diameter, $d = 3.0 \pm 0.2$ nm and a fitted shell thickness of $t = 2.2 \pm 0.2$ nm. These data are summarized in Table 3,

which also contains the solvent-core and solvent-shell scattering length density contrasts fitted from the SANS data: $\rho_{solv} - \rho_c = -0.57 \times 10^{-6} \text{ \AA}^{-2}$ while $\rho_{solv} - \rho_s$ is in the order of $3.0 \times 10^{-6} \text{ \AA}^{-2}$. This demonstrates that the shell has higher contrast than the iron oxide core, and therefore dominates the scattering signal of the core-shell particle. The absolute value of the shell scattering length density for 3.1 nm SL-IONP is $\rho_s = 3.45 \times 10^{-6} \text{ \AA}^{-2}$ and in good agreement with what is found for the 4.6 nm core SL-IONP.

Table 3. Structural parameters for the 3.1 nm and 4.6 nm SL-IONP obtained by combining TEM, SAXS and SANS analyses.

Sample	Technique	Core diameter d (nm)	Shell Thickness t (nm)	$\rho_{solv} - \rho_c$ ($\times 10^{-6} \text{ \AA}^{-2}$)	$\rho_{solv} - \rho_s$ ($\times 10^{-6} \text{ \AA}^{-2}$)
3.1 nm SL-IONP	TEM	3.1 ± 0.2	-	-	-
	SAXS	3.0 ± 0.4	-	-30.60	< -1.00
	SANS	3.0 ± 0.4	2.5 ± 0.2	-0.57	2.63
4.6 nm SL-IONP	TEM	4.6 ± 0.2	-	-	-
	SAXS	4.8 ± 0.4	-	-30.60	< -1.00
	SANS	4.8 ± 0.4	2.2 ± 0.2	-0.57	3.00

The scattering curve of the 14.1 nm SL-IONP sample was radically different to those of the dispersions of the two smaller SL-grafted IONP and is shown in Figure 5. The typical SANS spectrum for 14.1 nm SL-IONP in D₂O (blue filled circles) is relatively featureless in D₂O, but it shows strong scattering with a characteristic slope in the low- q region that is not present in the SANS spectra of the smaller SL-grafted IONP. This additional feature can be explained by comparing the data to the SANS spectrum of the pure SL-NDA ligand solution (Figure 5, grey filled circles), which nicely matches the spectrum of the 14.1 nm SL-IONP sample. Thus, in good agreement with the TGA data presented in Figure S4 and Table 2, the SANS signal of the 14.1 nm SL-IONP dispersion is dominated by SL-NDA in the form of filamentous structures instead of the core-shell particles. The higher contrast of SL-NDA to D₂O than of iron oxide to D₂O further contributes to the dominance of scattering from the SL-NDA micelles. These micelles were also prevalent in cryo-TEM images of the 14.1 nm SL-IONP sample (see Figure 3B). Nonetheless, SL-NDA is soluble in perdeuterated methanol, as shown by the corresponding poor SANS signal (grey empty

circles) in Figure 5 5. As expected, the SANS pattern of 14.1 nm SL-IONP (blue empty circles) in MeOD-d4 loses the SL-NDA scattering profile and it rather presents the typical feature of spherical objects. A shell-free 14.1 nm diameter IONP nanoparticle would be expected to have the first minimum of the form factor around $q = 0.4 \text{ nm}^{-1}$, whereas the minimum of the oscillation of the form factor measured for the 14.1 nm SL-IONP falls between $0.2 \text{ nm}^{-1} < q < 0.3 \text{ nm}^{-1}$, suggesting a spherical object sized between 20 and 30 nm. This is largely in agreement to what would be expected for a 14.1 nm iron oxide core, stabilized by a single SL-NDA layer even if the conditions of analysis (excess of SL-NDA, methanol) and likely presence of aggregates do not allow the quantification of the thickness of the SL-NDA layer.

Figure 5. SANS data recorded for 14.1 nm SL-IONP in D₂O (blue filled circles) and MeOD-d₄ (blue empty circles) and the pure ligand SL-NDA in D₂O (grey filled circles) and MeOD-d₄ (grey empty circles).

Colloidal stability and long-term stability. Removal of all monomeric free SL-NDA, is a good test of the colloidal stability of SL-NDA-grafted IONP. However, a biological environment is much more challenging for IONP colloidal stability, due to the higher concentration of biological colloids, displaying a larger variety of attractive interactions and leading to nanoparticle aggregation.⁵ Thus, colloidal stability of nanoparticles meant

1
2
3
4 to perform functions in biotechnological or medical applications should be tested under
5
6
7 conditions where they are exposed to full physiological ionic strength as well as to
8
9
10 concentrations of protein similar to those that could be encountered during application.
11
12
13 Therefore, we suspended the SL-IONP in 10 % fetal calf serum (FCS), which has a lower
14
15
16 concentration than full serum, but at which the colloidal stability can be monitored by DLS.
17
18
19 10 % serum also corresponds to the typical additive to cell culture media. We performed
20
21
22 the measurements while heating from 20 °C to 70 °C and back. These conditions are
23
24
25 known to weaken the hydration of the nanoparticle shell⁵⁵ and thereby reduce the colloidal
26
27
28 stability and decrease the ability to suppress adsorption of serum protein. Adsorption of
29
30
31 denatured proteins leads to irreversible aggregation and precipitation of insufficiently
32
33
34 stabilized nanoparticles⁵⁶. Figure 6 shows the evolution of the volume weighted
35
36
37 hydrodynamic diameter D_H of 4.6 nm SL-IONP in water with 10 % FCS with temperature.
38
39
40
41
42
43
44
45 Volume weighed D_H of 4.6 nm SL-IONP in water is shown in Figure S8A. The
46
47
48 hydrodynamic diameter of 4.6 nm SL-IONP was measured to be around 12 nm, which
49
50
51 was stable after more than 1.5 years in storage. The SL-IONP with 3.1 nm and 14.1 nm
52
53
54
55
56 core sizes appeared equally stable in dispersion over the same time period. However, the
57
58
59
60

1
2
3 stability could not be quantitatively confirmed by the hydrodynamic diameter, as there
4
5
6
7 were excess SL-NDA in these samples. All investigated SL-IONP have small diameters
8
9
10 in the superparamagnetic range and they are stabilized predominantly by steric and
11
12
13 hydration forces from the hydrophilic headgroup region of the sophorolipid. It is
14
15
16
17 advantageous for many applications to have a thin stabilizing shell of this kind to reduce
18
19
20 the overall hydrodynamic size. However, we note that the strength of the long-range
21
22
23 attractive van der Waals forces increases if the inorganic nanoparticle core is made
24
25
26
27 larger. It is likely that there is a maximum core size for which an SL-NDA shell can provide
28
29
30
31 sufficient stabilization against aggregation by dipolar interactions.
32
33

34
35 The hydrodynamic diameter of the 4.6 nm SL-IONP is in reasonable agreement with
36
37
38 the size of the core-shell nanoparticle determined by SANS, ~9.2 nm, which might
39
40
41 underestimate the steric or hydrodynamic size due to the fitting of a homogeneous shell.
42
43
44
45 The DLS result is also in good agreement with a theoretical size of a core-shell IONP
46
47
48 calculated from the diameter of the core measured by TEM (4.6 nm) and the length of a
49
50
51 SL-NDA molecule (3 nm), which implies a total steric diameter of 10.6 nm diameter. Thus,
52
53
54
55 both DLS and SANS data strongly suggest a monolayer of SL-NDA ligands on the NP
56
57
58
59
60

surface. The size distribution measured for the monodisperse iron oxide nanoparticles grafted with SL-NDA is narrower and with a smaller D_H than the SL-coated iron oxide nanoparticles reported in our earlier work.²⁶ This is attributed to the novel synthesis protocol with improved ligand and anchor chemistry for grafting to monodisperse iron oxide core particles. Volume weighted D_H size distributions of 10 % FCS in Milli-Q without nanoparticles measured at 20 °C and 70 °C are shown in Figure S8B. The size of serum proteins was measured to be ~5 nm at 20 °C. A size shift to 8 nm was observed at elevated temperatures.

Figure 6. Volume weighted hydrodynamic diameters D_H of 4.6 nm SL-IONP dispersed in water with 10 % FCS heated from A) 20 °C to 70 °C and B) cooled from 70 °C to 20 °C with 3 minutes equilibration time at each temperature.

When heating up the SL-IONP in FCS, we observed a size distribution with the main peak corresponding to the abundant serum albumin, with a tail towards larger sizes contributed by the SL-IONP. Upon heating, the fraction of small objects (serum albumin) in the size distribution decreased and the volume fraction of larger objects (nanoparticles) increased. This results from partial denaturation and aggregation of serum proteins. This trend becomes even more obvious in Figure 6B where a temperature of 70 °C was held for 8 minutes to allow for more equilibration of the sample. Here, the apparent volume fraction of nanoparticles is even larger, which could be due to precipitation of serum protein. Importantly, no aggregation and precipitation of SL-IONP was observed at any temperature, with or without exposure to denatured serum protein. This can also be observed from the intensity weighted size distributions for the same temperature cycling experiment (Figure S11). Figure S9 shows a photograph of SL-IONP after temperature cycling. Aggregation of SL-IONP could not be induced by heating the samples in FCS at 70 °C for 3 h (Figure S10). Similar to the observations during temperature cycling, only a

1
2
3 small shift in size is observed after heating for 3 h and a very small peak in the intensity
4
5
6
7 weighted D_H appears at large sizes, indicating the aggregation of the protein that
8
9
10 precipitated after 3 h heating (Figure S12). These data, showing the excellent stability of
11
12
13 SL-IONP with temperature, can be explained by the small influence that temperature has
14
15
16 on the dehydration of the carbohydrate moiety of sugar-based capping agents. Sugar-
17
18
19 based dispersants are both hydrogen-bond donors and acceptors, which allows for a bulk
20
21
22 water-like hydrogen-bonding network and thereby low sensitivity to dehydration upon
23
24
25 increasing temperature.
26
27
28
29
30

31 The excellent colloidal stability of the SL-IONP nanoparticles is further demonstrated by
32
33
34 additional experiments performed in the presence of 1 M NaCl in D₂O. The high ionic
35
36
37 strength of 1 M NaCl was chosen for the ability of the NaCl ions to screen and reduce
38
39
40 double layer repulsion, thereby increasing the propensity of nanoparticles to aggregate,
41
42
43 as well as the ability of the mild kosmotropes to reduce hydration and thereby decrease
44
45
46 stabilization due to steric and hydration forces. Figure 7 presents the typical SANS
47
48
49 spectra for the 3.1 nm and 4.6 nm SL-IONP in 1 M NaCl D₂O in comparison with SL-IONP
50
51
52
53
54
55
56 in pure D₂O.
57
58
59
60

Figure 7. SANS data recorded for 3.1 nm and 4.6 nm SL-IONP in D₂O (red and grey filled circles respectively) and in 1 M NaCl (dissolved in D₂O) (red and grey empty circles respectively).

The data show that the SANS profiles of neither sample change when the ionic strength of the solvent is increased. The absence of change is true in both the mid/high- q region characteristic of the core-shell structure and in the low- q region characteristic of aggregation. This indicates that the SL-NDA shell has neither been removed upon salt addition, nor has its structure been modified. This contrasts to previous findings for

sophorolipid coated nanoparticles, where aggregation was promoted by the addition of salt.²⁶ The improved stability of our novel SL-IONP can be attributed to the NDA anchor chemistry that irreversibly grafts the SL coating densely to the cores. Furthermore, the SANS profiles do not show any sign of particle aggregation, since a structure factor corresponding to particle aggregation would appear in the low q -range. There is also no optically visible increase in scattering nor does precipitation take place, which supports the SANS observation of excellent colloidal stability. This is in full agreement with the DLS data that showed retained size and no aggregation and precipitation (*cf.* Figure 6). The SANS measurements also show that micelles of excess ligand in the 3.1 nm SL-IONP sample were not altered by that addition of salt (no change in the slope at low q), which explains why dialysis at high ionic strength could not remove the excess ligand.

***In-vitro* cellular uptake and cytotoxicity of SL-IONP.** To prove the applicability of SL-IONP in the biomedical field, we conducted cellular uptake and cytotoxicity tests on two different cell lines: U937 monocytes which are part of the reticuloendothelial system and

1
2
3 responsible for clearance of pathogens and foreign bodies from the blood and MCF7, a
4
5
6
7 human breast cancer cell line.
8
9

10 Differentiated monocytes, macrophages, are the cells in the body that are most exposed
11
12
13
14 to toxins and foreign bodies. The response of these cells to exposure of SL-IONP is thus
15
16
17
18 of highest relevance. If they detect and scavenge nanoparticles, the nanoparticles will be
19
20
21 of limited use in applications that require circulation and/or targeting. It is, to the best of
22
23
24 our knowledge, the first time that cellular uptake and toxicity are studied for sophorolipid
25
26
27
28 coated core-shell nanoparticles. Figure 8 shows the results of the cellular uptake and
29
30
31 cytotoxicity tests for 4.6 nm SL-IONP with U937 and MCF7 cells. SL-IONP show very low
32
33
34
35 non-specific uptake by both monocytes and cancer cells. A slight trend to higher uptake
36
37
38 of breast cancer cells over the phagocytic monocytes could not be statistically verified.
39
40
41
42 The low uptake is in the same order as previously shown for stealth poly(ethylene glycol)-
43
44
45 grafted IONP⁹ and can be traced back to the good colloidal stability of the core-shell SL-
46
47
48
49 IONP and their resistance against aggregation in presence of proteins and salts (*cf.*
50
51
52 Figure 6 and 7).
53
54
55
56
57
58
59
60

Both sphorolipids and iron oxide nanoparticles have individually shown negligible toxicity at relevant concentrations to various cell lines.⁵⁷⁻⁵⁸ Figure 8B shows that also SL-grafted 4.6 nm IONP do not affect the viability of the two tested cell lines. The results suggest an excellent biocompatibility of the SL-IONP.

Figure 8. A) Cellular uptake of 4.6 nm SL-IONP for human monocytes (U937) and human breast cancer cells (MCF7). Negligible uptake was observed with a tendency towards increased uptake in cancer cell lines, but without statistical significance ($p > 0.05$ ANOVA 1-way) within the study. B) Cytotoxicity tests for 4.6 nm SL-IONP with human monocytes (U937) and human breast cancer cells (MCF7). The viability was unchanged for both cell lines.

Design principles for using SL-NDA to stabilize IONP for applications. In summary, a range of challenging conditions for colloidal stability relevant for applications were tested; all results were consistent with excellent stability of the grafted SL-NDA shell translating to equally excellent colloidal stability. The low size dispersity, high grafting density and colloidal stability of the SL-IONP system developed here are comparable to those of synthetic glyconanoparticles^{12, 16-17, 59-60} rather than sophorolipid-capped nanoparticles.²³⁻²⁶ Our results demonstrate that the densely grafted SL-NDA shell provides a stable repulsive potential preventing both mutual aggregation and protein adsorption under highly challenging conditions (1 M NaCl, 70 °C with and without FCS). The colloidal stability is not adversely affected by temperature and kosmotrope salts that often have a destabilizing effect on polymer coated-core-shell nanoparticles. This is reached by the irreversible grafting of the SL ligand to the core in contrast to pure self-assembly and/or complexation. The expected main contribution to the repulsive interaction of the shell is the high and stable hydration of the lipid head-groups that is apparently strong enough to withstand weakening in the coordination of associated water under relevant challenging

environmental conditions. This fact is confirmed by the literature on the micellization properties of sugar-based amphiphiles: the strength between the hydrogen bonding between the hydroxyl groups of the sugar unit and water is stronger than between ethylene oxide and water.⁶¹

It is worth noting that stabilization through a grafted lipid is fundamentally different to the more commonly applied approach of a high- M_w flexible polymer; the low thickness added by a lipid monolayer and its insignificant molecular flexibility makes it possible to design such shells much more compact and structurally precise than using solvated polymer. At the same time, it is impressive that equally high colloidal stability, as demonstrated here, can be obtained. The hydrophobic spacer part of the lipid shell is important to reduce core-core van-der-Waals attraction and likely cannot be made too thin without compromising colloidal stability. However, with a hydrophobic interior of the shell, a prerequisite for stability in biofluids is that the lipid shell (the lipid headgroup region) is dense enough to prevent hydrophobic molecules, such as protein denatured by temperature, to adsorb to shell defects exposing the alkyl part of the lipids. We achieved this by the stable NDA-coupling and efficient ligand exchange protocol used to graft the

1
2
3 SL-NDA shell. As expected, in the absence of aggregates, the SL-IONP showed only
4
5
6
7 weak interaction with cells *in-vitro* with negligible uptake and no resulting cytotoxicity, thus
8
9
10 making these systems promising candidates for the development of IONP-based
11
12
13 biomedical applications. These, so-called stealth, nanoparticles can be further
14
15
16
17 functionalized or make use of specific interactions of the exposed saccharide motif to add
18
19
20 targeting capability, for which structural and colloidal stability, as well as suppression of
21
22
23 non-specific biomolecular interactions, is a prerequisite.
24
25
26
27
28
29
30

31 Conclusion

32
33
34 We have synthesized monodisperse, biocompatible, sophorolipid-grafted core-shell
35
36
37 iron oxide nanoparticles with excellent colloidal stability in demanding application
38
39
40 environments mimicking the conditions of biological fluids. As-synthesized, oleic acid-
41
42
43 coated IONP with precisely tunable sizes and narrow size distributions were successfully
44
45
46
47 grafted with a nitrodopamide-modified sophorolipid ligand by replacement of the oleic
48
49
50 acid. Analysis of TGA and cryo-TEM highlighted the challenge of removing excess ligand
51
52
53
54
55
56 as sophorolipid forms very stable aggregates of similar size as the grafted nanoparticles
57
58
59
60

1
2
3 and with the same physico-chemical characteristics. Cryo-TEM revealed the coexistence
4
5
6 of individual, monodisperse, SL-grafted IONP with large and stable SL-NDA aggregates
7
8
9 formed by excess ligand that could not be completely removed by excessive purification.
10
11
12
13 However, also close to pure SL-grafted IONP product was obtained for 4.6 nm IONP
14
15
16
17 cores. The core-shell structure of SL-IONP was analyzed by SAXS and SANS. The
18
19
20
21 resulting data were successfully fitted with a core-shell model. SANS revealed the
22
23
24 thickness of the shell, which was found to be in the order of 2.5 nm, in good agreement
25
26
27 with a shell composed of a monolayer of SL-NDA grafted to a highly curved surface.
28
29
30

31 The stable, grafted core-shell architecture presenting the equivalent of a dense
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65
66
67
68
69
70
71
72
73
74
75
76
77
78
79
80
81
82
83
84
85
86
87
88
89
90
91
92
93
94
95
96
97
98
99
100
101
102
103
104
105
106
107
108
109
110
111
112
113
114
115
116
117
118
119
120
121
122
123
124
125
126
127
128
129
130
131
132
133
134
135
136
137
138
139
140
141
142
143
144
145
146
147
148
149
150
151
152
153
154
155
156
157
158
159
160
161
162
163
164
165
166
167
168
169
170
171
172
173
174
175
176
177
178
179
180
181
182
183
184
185
186
187
188
189
190
191
192
193
194
195
196
197
198
199
200
201
202
203
204
205
206
207
208
209
210
211
212
213
214
215
216
217
218
219
220
221
222
223
224
225
226
227
228
229
230
231
232
233
234
235
236
237
238
239
240
241
242
243
244
245
246
247
248
249
250
251
252
253
254
255
256
257
258
259
260
261
262
263
264
265
266
267
268
269
270
271
272
273
274
275
276
277
278
279
280
281
282
283
284
285
286
287
288
289
290
291
292
293
294
295
296
297
298
299
300
301
302
303
304
305
306
307
308
309
310
311
312
313
314
315
316
317
318
319
320
321
322
323
324
325
326
327
328
329
330
331
332
333
334
335
336
337
338
339
340
341
342
343
344
345
346
347
348
349
350
351
352
353
354
355
356
357
358
359
360
361
362
363
364
365
366
367
368
369
370
371
372
373
374
375
376
377
378
379
380
381
382
383
384
385
386
387
388
389
390
391
392
393
394
395
396
397
398
399
400
401
402
403
404
405
406
407
408
409
410
411
412
413
414
415
416
417
418
419
420
421
422
423
424
425
426
427
428
429
430
431
432
433
434
435
436
437
438
439
440
441
442
443
444
445
446
447
448
449
450
451
452
453
454
455
456
457
458
459
460
461
462
463
464
465
466
467
468
469
470
471
472
473
474
475
476
477
478
479
480
481
482
483
484
485
486
487
488
489
490
491
492
493
494
495
496
497
498
499
500
501
502
503
504
505
506
507
508
509
510
511
512
513
514
515
516
517
518
519
520
521
522
523
524
525
526
527
528
529
530
531
532
533
534
535
536
537
538
539
540
541
542
543
544
545
546
547
548
549
550
551
552
553
554
555
556
557
558
559
560
561
562
563
564
565
566
567
568
569
570
571
572
573
574
575
576
577
578
579
580
581
582
583
584
585
586
587
588
589
590
591
592
593
594
595
596
597
598
599
600
601
602
603
604
605
606
607
608
609
610
611
612
613
614
615
616
617
618
619
620
621
622
623
624
625
626
627
628
629
630
631
632
633
634
635
636
637
638
639
640
641
642
643
644
645
646
647
648
649
650
651
652
653
654
655
656
657
658
659
660
661
662
663
664
665
666
667
668
669
670
671
672
673
674
675
676
677
678
679
680
681
682
683
684
685
686
687
688
689
690
691
692
693
694
695
696
697
698
699
700
701
702
703
704
705
706
707
708
709
710
711
712
713
714
715
716
717
718
719
720
721
722
723
724
725
726
727
728
729
730
731
732
733
734
735
736
737
738
739
740
741
742
743
744
745
746
747
748
749
750
751
752
753
754
755
756
757
758
759
760
761
762
763
764
765
766
767
768
769
770
771
772
773
774
775
776
777
778
779
780
781
782
783
784
785
786
787
788
789
790
791
792
793
794
795
796
797
798
799
800
801
802
803
804
805
806
807
808
809
810
811
812
813
814
815
816
817
818
819
820
821
822
823
824
825
826
827
828
829
830
831
832
833
834
835
836
837
838
839
840
841
842
843
844
845
846
847
848
849
850
851
852
853
854
855
856
857
858
859
860
861
862
863
864
865
866
867
868
869
870
871
872
873
874
875
876
877
878
879
880
881
882
883
884
885
886
887
888
889
890
891
892
893
894
895
896
897
898
899
900
901
902
903
904
905
906
907
908
909
910
911
912
913
914
915
916
917
918
919
920
921
922
923
924
925
926
927
928
929
930
931
932
933
934
935
936
937
938
939
940
941
942
943
944
945
946
947
948
949
950
951
952
953
954
955
956
957
958
959
960
961
962
963
964
965
966
967
968
969
970
971
972
973
974
975
976
977
978
979
980
981
982
983
984
985
986
987
988
989
990
991
992
993
994
995
996
997
998
999
1000
1001
1002
1003
1004
1005
1006
1007
1008
1009
1010
1011
1012
1013
1014
1015
1016
1017
1018
1019
1020
1021
1022
1023
1024
1025
1026
1027
1028
1029
1030
1031
1032
1033
1034
1035
1036
1037
1038
1039
1040
1041
1042
1043
1044
1045
1046
1047
1048
1049
1050
1051
1052
1053
1054
1055
1056
1057
1058
1059
1060
1061
1062
1063
1064
1065
1066
1067
1068
1069
1070
1071
1072
1073
1074
1075
1076
1077
1078
1079
1080
1081
1082
1083
1084
1085
1086
1087
1088
1089
1090
1091
1092
1093
1094
1095
1096
1097
1098
1099
1100
1101
1102
1103
1104
1105
1106
1107
1108
1109
1110
1111
1112
1113
1114
1115
1116
1117
1118
1119
1120
1121
1122
1123
1124
1125
1126
1127
1128
1129
1130
1131
1132
1133
1134
1135
1136
1137
1138
1139
1140
1141
1142
1143
1144
1145
1146
1147
1148
1149
1150
1151
1152
1153
1154
1155
1156
1157
1158
1159
1160
1161
1162
1163
1164
1165
1166
1167
1168
1169
1170
1171
1172
1173
1174
1175
1176
1177
1178
1179
1180
1181
1182
1183
1184
1185
1186
1187
1188
1189
1190
1191
1192
1193
1194
1195
1196
1197
1198
1199
1200
1201
1202
1203
1204
1205
1206
1207
1208
1209
1210
1211
1212
1213
1214
1215
1216
1217
1218
1219
1220
1221
1222
1223
1224
1225
1226
1227
1228
1229
1230
1231
1232
1233
1234
1235
1236
1237
1238
1239
1240
1241
1242
1243
1244
1245
1246
1247
1248
1249
1250
1251
1252
1253
1254
1255
1256
1257
1258
1259
1260
1261
1262
1263
1264
1265
1266
1267
1268
1269
1270
1271
1272
1273
1274
1275
1276
1277
1278
1279
1280
1281
1282
1283
1284
1285
1286
1287
1288
1289
1290
1291
1292
1293
1294
1295
1296
1297
1298
1299
1300
1301
1302
1303
1304
1305
1306
1307
1308
1309
1310
1311
1312
1313
1314
1315
1316
1317
1318
1319
1320
1321
1322
1323
1324
1325
1326
1327
1328
1329
1330
1331
1332
1333
1334
1335
1336
1337
1338
1339
1340
1341
1342
1343
1344
1345
1346
1347
1348
1349
1350
1351
1352
1353
1354
1355
1356
1357
1358
1359
1360
1361
1362
1363
1364
1365
1366
1367
1368
1369
1370
1371
1372
1373
1374
1375
1376
1377
1378
1379
1380
1381
1382
1383
1384
1385
1386
1387
1388
1389
1390
1391
1392
1393
1394
1395
1396
1397
1398
1399
1400
1401
1402
1403
1404
1405
1406
1407
1408
1409
1410
1411
1412
1413
1414
1415
1416
1417
1418
1419
1420
1421
1422
1423
1424
1425
1426
1427
1428
1429
1430
1431
1432
1433
1434
1435
1436
1437
1438
1439
1440
1441
1442
1443
1444
1445
1446
1447
1448
1449
1450
1451
1452
1453
1454
1455
1456
1457
1458
1459
1460
1461
1462
1463
1464
1465
1466
1467
1468
1469
1470
1471
1472
1473
1474
1475
1476
1477
1478
1479
1480
1481
1482
1483
1484
1485
1486
1487
1488
1489
1490
1491
1492
1493
1494
1495
1496
1497
1498
1499
1500
1501
1502
1503
1504
1505
1506
1507
1508
1509
1510
1511
1512
1513
1514
1515
1516
1517
1518
1519
1520
1521
1522
1523
1524
1525
1526
1527
1528
1529
1530
1531
1532
1533
1534
1535
1536
1537
1538
1539
1540
1541
1542
1543
1544
1545
1546
1547
1548
1549
1550
1551
1552
1553
1554
1555
1556
1557
1558
1559
1560
1561
1562
1563
1564
1565
1566
1567
1568
1569
1570
1571
1572
1573
1574
1575
1576
1577
1578
1579
1580
1581
1582
1583
1584
1585
1586
1587
1588
1589
1590
1591
1592
1593
1594
1595
1596
1597
1598
1599
1600
1601
1602
1603
1604
1605
1606
1607
1608
1609
1610
1611
1612
1613
1614
1615
1616
1617
1618
1619
1620
1621
1622
1623
1624
1625
1626
1627
1628
1629
1630
1631
1632
1633
1634
1635
1636
1637
1638
1639
1640
1641
1642
1643
1644
1645
1646
1647
1648
1649
1650
1651
1652
1653
1654
1655
1656
1657
1658
1659
1660
1661
1662
1663
1664
1665
1666
1667
1668
1669
1670
1671
1672
1673
1674
1675
1676
1677
1678
1679
1680
1681
1682
1683
1684
1685
1686
1687
1688
1689
1690
1691
1692
1693
1694
1695
1696
1697
1698
1699
1700
1701
1702
1703
1704
1705
1706
1707
1708
1709
1710
1711
1712
1713
1714
1715
1716
1717
1718
1719
1720
1721
1722
1723
1724
1725
1726
1727
1728
1729
1730
1731
1732
1733
1734
1735
1736
1737
1738
1739
1740
1741
1742
1743
1744
1745
1746
1747
1748
1749
1750
1751
1752
1753
1754
1755
1756
1757
1758
1759
1760
1761
1762
1763
1764
1765
1766
1767
1768
1769
1770
1771
1772
1773
1774
1775
1776
1777
1778
1779
1780
1781
1782
1783
1784
1785
1786
1787
1788
1789
1790
1791
1792
1793
1794
1795
1796
1797
1798
1799
1800
1801
1802
1803
1804
1805
1806
1807
1808
1809
1810
1811
1812
1813
1814
1815
1816
1817
1818
1819
1820
1821
1822
1823
1824
1825
1826
1827
1828
1829
1830
1831
1832
1833
1834
1835
1836
1837
1838
1839
1840
1841
1842
1843
1844
1845
1846
1847
1848
1849
1850
1851
1852
1853
1854
1855
1856
1857
1858
1859
1860
1861
1862
1863
1864
1865
1866
1867
1868
1869
1870
1871
1872
1873
1874
1875
1876
1877
1878
1879
1880
1881
1882
1883
1884
1885
1886
1887
1888
1889
1890
1891
1892
1893
1894
1895
1896
1897
1898
1899
1900
1901
1902
1903
1904
1905
1906
1907
1908
1909
1910
1911
1912
1913
1914
1915
1916
1917
1918
1919
1920
1921
1922
1923
1924
1925
1926
1927
1928
1929
1930
1931
1932
1933
1934
1935
1936
1937
1938
1939
1940
1941
1942
1943
1944
1945
1946
1947
1948
1949
1950
1951
1952
1953
1954
1955
1956
1957
1958
1959
1960
1961
1962
1963
1964
1965
1966
1967
1968
1969
1970
1971
1972
1973
1974
1975
1976
1977
1978
1979
1980
1981
1982
1983
1984
1985
1986
1987
1988
1989
1990
1991
1992
1993
1994
1995
1996
1997
1998
1999
2000
2001
2002
2003
2004
2005
2006
2007
2008
2009
2010
2011
2012
2013
2014
2015
2016
2017
2018
2019
2020
2021
2022
2023
2024
2025
2026
2027
2028
2029
2030
2031
2032
2033
2034
2035
2036
2037
2038
2039
2040
2041
2042
2043
2044
2045
2046
2047
2048
2049
2050
2051
2052
2053
2054
2055
2056
2057
2058
2059
2060
2061
2062
2063
2064
2065
2066
2067
2068
2069
2070
2071
2072
2073
2074
2075
2076
2077
2078
2079
2080
2081
2082
2083
2084
2085
2086
2087
2088
2089
2090
2091
2092
2093
2094
2095
2096
2097
2098
2099
2100
2101
2102
2103
2104
2105
2106
2107
2108
2109
2110
2111
2112
2113
2114
2115
2116
2117
2118
2119
2120
2121
2122
2123
2124
2125
2126
2127
2128
2129
2130
2131
2132
2133
2134
2135
2136
2137
2138
2139
2140
2141
2142
2143
2144
2145
2146
2147
2148
2149
2150
2151
2152
2153
2154
2155
2156
2157
2158
2159
2160
2161
2162
2163
2164
2165
2166
2167
2168
2169
2170
2171
2172
2173
2174
2175
2176
2177
2178
2179
2180
2181
2182
2183
2184
2185
2186
2187
2188
2189
2190
2191
2192
2193
2194
2195
2196
2197
2198

nanoparticles can display unique targeting ability towards lectin-rich biological membranes, including tumor cells and pathogenic bacteria.

ASSOCIATED CONTENT

Supporting Information. Additional information on Materials. Control over iron oxide core size by the precursor to surfactant ratio. Detailed synthetic protocols. NMR, IR, TGA, TEM, cryo-TEM and DLS data for SL-NDA and SL-IONP. Photos of colloidal suspensions. Additional SAXS and SANS data with fits.

AUTHOR INFORMATION

Corresponding Authors

*Erik Reimhult: erik.reimhult@boku.ac.at; Niki Baccile: niki.baccile@upmc.fr.

Author Contributions

AL, ER and NB planned the work. AL and NB performed most of the experiments. AL, ER and NB wrote the manuscript. All authors contributed to and reviewed the manuscript.

1
2
3
4 The manuscript was written through contributions of all authors. All authors have given
5
6
7 approval to the final version of the manuscript.
8
9

10 11 **Funding Sources** 12 13

14
15 This work received financial support by the Institut Laue-Langevin (ILL), Grenoble,
16
17
18 France, under the experiment number 9-12-481 (D33, D16 beamlines), and by the
19
20
21 European Synchrotron Radiation Facility (ESRF), Grenoble, France, under the
22
23
24
25 experiment number SC-4419. The research presented in that paper was funded by the
26
27
28
29 Austrian Science Fund (FWF) project I 3064 and by the European Research Council
30
31
32 under the European Union's Seventh Framework Program (FP/2007-2013)/ERC Grant
33
34
35
36 Agreement No. 310034.
37
38
39

40 **ACKNOWLEDGMENT** 41 42 43 44

45 This work benefited from the use of the SasView application, originally developed under
46
47
48 NSF award DMR-0520547. SasView contains code developed with funding from the
49
50
51 European Union's Horizon 2020 research and innovation programme under the
52
53
54
55 SINE2020 project, grant agreement No 654000. We thank the Lawrence Berkeley
56
57
58
59
60

National Laboratory for access to TEM; work at the Molecular Foundry was supported by the Office of Science, Office of Basic Energy Sciences, of the U.S. Department of Energy under Contract No. DE-AC02-05CH11231. We thank Prof. Dieter Baurecht for access to FTIR. We acknowledge the VIBT Extremophile Center for access to TGA.

ABBREVIATIONS

IONP, iron oxide nanoparticles; OA, oleic acid; Oct₂O, dioctylether; SL, sophorolipids; NDA, nitrodopamine/nitrodopamide; SL-NDA, sophorolipid-nitrodopamide; SL-IONP, sophorolipid-nitrodopamide-grafted iron oxide nanoparticles; PEG, poly(ethylene glycol); PBS, Phosphate buffered saline; FCS, fetal calf serum; HR, high-resolution; LR, low-resolution; TEM, transmission electron microscope/microscopy; TGA, thermogravimetric analysis; DLS, dynamic light scattering; NMR, nuclear magnetic resonance; ATR-FTIR, attenuated total reflection – Fourier transform infra-red; SAXS, small angle x-ray scattering; SANS, small angle neutron scattering; SLD, scattering length density; MWCO, molecular weight cut-off; TOC, total organic content

REFERENCES

1. Hong, R. Y.; Feng, B.; Chen, L. L.; Liu, G. H.; Li, H. Z.; Zheng, Y.; Wei, D. G., Synthesis, Characterization and MRI Application of Dextran-Coated Fe₃O₄ Magnetic Nanoparticles. *Biochem. Eng. J.* 2008, 42, 290-300.
2. Lacava, L. M.; Lacava, Z. G. M.; Da Silva, M. F.; Silva, O.; Chaves, S. B.; Azevedo, R. B.; Pelegrini, F.; Gansau, C.; Buske, N.; Sabolovic, D.; Morais, P. C., Magnetic Resonance of a Dextran-Coated Magnetic Fluid Intravenously Administered in Mice. *Biophys. J.* 2001, 80, 2483-2486.
3. Tassa, C.; Shaw, S. Y.; Weissleder, R., Dextran-Coated Iron Oxide Nanoparticles: A Versatile Platform for Targeted Molecular Imaging, Molecular Diagnostics, and Therapy. *Acc. Chem. Res.* 2011, 44, 842-852.
4. Boni, A.; Bardi, G.; Bertero, A.; Cappello, V.; Emdin, M.; Flori, A.; Gemmi, M.; Innocenti, C.; Menichetti, L.; Sangregorio, C.; Villa, S.; Piazza, V., Design and Optimization of Lipid-Modified Poly(Amidoamine) Dendrimer Coated Iron Oxide Nanoparticles as Probes for Biomedical Applications. *Nanoscale* 2015, 7, 7307-7317.
5. Amstad, E.; Textor, M.; Reimhult, E., Stabilization and Functionalization of Iron Oxide Nanoparticles for Biomedical Applications. *Nanoscale* 2011, 3, 2819-2843.
6. Frey, N. A.; Peng, S.; Cheng, K.; Sun, S., Magnetic Nanoparticles: Synthesis, Functionalization, and Applications in Bioimaging and Magnetic Energy Storage. *Chem. Soc. Rev.* 2009, 38, 2532-2542.
7. Park, J.; Joo, J.; Kwon, S. G.; Jang, Y.; Hyeon, T., Synthesis of Monodisperse Spherical Nanocrystals. *Angew. Chem. Int. Ed.* 2007, 46, 4630-4660.
8. Lassenberger, A.; Bixner, O.; Gruenewald, T.; Lichtenegger, H.; Zirbs, R.; Reimhult, E., Evaluation of High-Yield Purification Methods on Monodisperse Peg-Grafted Iron Oxide Nanoparticles. *Langmuir* 2016, 32, 4259-4269.
9. Lassenberger, A.; Scheberl, A.; Stadlbauer, A.; Stiglbauer, A.; Helbich, T.; Reimhult, E., Individually Stabilized, Superparamagnetic Nanoparticles with Controlled Shell and Size Leading to Exceptional Stealth Properties and High Relaxivities. *ACS Appl. Mater. Interfaces* 2017, 9, 3343-3353.

10. Yang, Q.; Lai, S. K., Anti-PEG Immunity: Emergence, Characteristics, and Unaddressed Questions. Wiley interdisciplinary reviews. Nanomed. Nanobiotechnol. 2015, 7, 655-677.
11. Bernardi, A.; Jimenez-Barbero, J.; Casnati, A.; De Castro, C.; Darbre, T.; Fieschi, F.; Finne, J.; Funken, H.; Jaeger, K.-E.; Lahmann, M.; Lindhorst, T. K.; Marradi, M.; Messner, P.; Molinaro, A.; Murphy, P. V.; Nativi, C.; Oscarson, S.; Penades, S.; Peri, F.; Pieters, R. J.; Renaudet, O.; Reymond, J.-L.; Richichi, B.; Rojo, J.; Sansone, F.; Schaffer, C.; Turnbull, W. B.; Velasco-Torrijos, T.; Vidal, S.; Vincent, S.; Wennekes, T.; Zuilhof, H.; Imberty, A., Multivalent Glycoconjugates as Anti-Pathogenic Agents. Chem. Soc. Rev. 2013, 42, 4709-4727.
12. Marradi, M.; Chiodo, F.; Garcia, I.; Penades, S., Glyconanoparticles as Multifunctional and Multimodal Carbohydrate Systems. Chem. Soc. Rev. 2013, 42, 4728-4745.
13. Lee, Y. C.; Lee, R. T., Carbohydrate-Protein Interactions: Basis of Glycobiology. Acc. Chem. Res. 1995, 28, 321-327.
14. Lis, H.; Sharon, N., Lectins: Carbohydrate-Specific Proteins That Mediate Cellular Recognition. Chem. Rev. 1998, 98, 637-674.
15. Cecioni, S.; Imberty, A.; Vidal, S., Glycomimetics Versus Multivalent Glycoconjugates for the Design of High Affinity Lectin Ligands. Chem. Rev. 2015, 115, 525-561.
16. El-Boubbou, K.; Zhu, D. C.; Vasileiou, C.; Borhan, B.; Prosperi, D.; Li, W.; Huang, X., Magnetic Glyco-Nanoparticles: A Tool to Detect, Differentiate, and Unlock the Glyco-Codes of Cancer Via Magnetic Resonance Imaging. J. Am. Chem. Soc. 2010, 132, 4490-4499.
17. Gallo, J.; Garcia, I.; Padro, D.; Arnaiz, B.; Penades, S., Water-Soluble Magnetic Glyconanoparticles Based on Metal-Doped Ferrites Coated with Gold: Synthesis and Characterization. J. Mater. Chem. B 2010, 20, 10010-10020.
18. Dumur, F.; Guerlin, A.; Dumas, E.; Bertin, D.; Gigmes, D.; Mayer, C. R., Controlled Spontaneous Generation of Gold Nanoparticles Assisted by Dual Reducing and Capping Agents. Gold Bulletin 2011, 44, 119-137.

19. Duan, H.; Wang, D.; Li, Y., Green Chemistry for Nanoparticle Synthesis. *Chem. Soc. Rev.* 2015, 44, 5778-5792.
20. Manet, S.; Cuvier, A.-S.; Valotteau, C.; Fadda, G. C.; Perez, J.; Karakas, E.; Abel, S.; Baccile, N., Structure of Bolaamphiphile Sophorolipid Micelles Characterized with SAXS, SANS, and MD Simulations. *J. Phys. Chem. B* 2015, 119, 13113-13133.
21. Chen, J.; Song, X.; Zhang, H.; Qu, Y., Production, Structure Elucidation and Anticancer Properties of Sophorolipid from *Wickerhamiella Domercqiae*. *Enzyme Microb. Technol.* 2006, 39, 501-506.
22. Díaz De Rienzo, M. A.; Banat, I. M.; Dolman, B.; Winterburn, J.; Martin, P. J., Sophorolipid Biosurfactants: Possible Uses as Antibacterial and Antibiofilm Agent. *New Biotechnol.* 2015, 32, 720-726.
23. Kasture, M.; Singh, S.; Patel, P.; Joy, P. A.; Prabhune, A. A.; Ramana, C. V.; Prasad, B. L. V., Multiutility Sophorolipids as Nanoparticle Capping Agents: Synthesis of Stable and Water Dispersible Co Nanoparticles. *Langmuir* 2007, 23, 11409-11412.
24. Singh, S.; Patel, P.; Jaiswal, S.; Prabhune, A. A.; Ramana, C. V.; Prasad, B. L. V., A Direct Method for the Preparation of Glycolipid-Metal Nanoparticle Conjugates: Sophorolipids as Reducing and Capping Agents for the Synthesis of Water Re-Dispersible Silver Nanoparticles and Their Antibacterial Activity. *New J. Chem.* 2009, 33, 646-652.
25. Kumar, D. V. R.; Kasture, M.; Prabhune, A. A.; Ramana, C. V.; Prasad, B. L. V.; Kulkarni, A. A., Continuous Flow Synthesis of Functionalized Silver Nanoparticles Using Bifunctional Biosurfactants. *Green Chem.* 2010, 12, 609-615.
26. Baccile, N.; Noiville, R.; Stievano, L.; Bogaert, I. V., Sophorolipids-Functionalized Iron Oxide Nanoparticles. *Phys. Chem. Chem. Phys.* 2013, 15, 1606-1620.
27. Amstad, E.; Gehring, A. U.; Fischer, H.; Nagaiyanallur, V. V.; Hahner, G.; Textor, M.; Reimhult, E., Influence of Electronegative Substituents on the Binding Affinity of Catechol-Derived Anchors to Fe₃O₄ Nanoparticles. *J. Phys. Chem. C* 2011, 115, 683-691.
28. Amstad, E.; Gillich, T.; Bilecka, I.; Textor, M.; Reimhult, E., Ultrastable Iron Oxide Nanoparticle Colloidal Suspensions Using Dispersants with Catechol-Derived Anchor Groups. *Nano Letters* 2009, 9, 4042-4048.

29. Baccile, N.; Cuvier, A.-S.; Valotteau, C.; Van Bogaert, I. N. A., Practical Methods to Reduce Impurities for Gram-Scale Amounts of Acidic Sophorolipid Biosurfactants. *Eur. J. Lipid Sci. Technol.* 2013, 115, 1404-1412.
30. Dhasaiyan, P.; Le Griel, P.; Roelants, S.; Redant, E.; Van Bogaert, I. N. A.; Prevost, S.; Prasad, B. L. V.; Baccile, N., Micelles Versus Ribbons: How Congeners Drive the Self-Assembly of Acidic Sophorolipid Biosurfactants. *Chem. Phys. Chem.* 2017, 18, 643-652.
31. Napolitano, A.; D'Ischia, M.; Costantini, C.; Prota, G., A New Oxidation Pathway of the Neurotoxin 6-Aminodopamine. Isolation and Characterization of a Dimer with a Tetrahydro[3,4a]Iminoethanophenoxazine Ring System. *Tetrahedron* 1992, 48, 8515-8522.
32. Bixner, O.; Lassenberger, A.; Baurecht, D.; Reimhult, E., Complete Exchange of the Hydrophobic Dispersant Shell on Monodisperse Superparamagnetic Iron Oxide Nanoparticles. *Langmuir* 2015, 31, 9198-9204.
33. El-Faham, A.; Funosas, R. S.; Prohens, R.; Albericio, F., Comu: A Safer and More Effective Replacement for Benzotriazole-Based Uronium Coupling Reagents. *Chem. - Eur. J.* 2009, 15, 9404-9416.
34. Kurzahls, S.; Zirbs, R.; Reimhult, E., Synthesis and Magneto-Thermal Actuation of Iron Oxide Core–Pnlpam Shell Nanoparticles. *ACS App. Mater. Interfaces* 2015, 7, 19342-19352.
35. Hyeon, T.; Lee, S. S.; Park, J.; Chung, Y.; Na, H. B., Synthesis of Highly Crystalline and Monodisperse Maghemite Nanocrystallites without a Size-Selection Process. *J. Am. Chem. Soc.* 2001, 123, 12798-12801.
36. Park, J.; An, K.; Hwang, Y.; Park, J.-G.; Noh, H.-J.; Kim, J.-Y.; Park, J.-H.; Hwang, N.-M.; Hyeon, T., Ultra-Large-Scale Syntheses of Monodisperse Nanocrystals. *Nat. Mater.* 2004, 3, 891-895.
37. Mondini, S.; Ferretti, A. M.; Puglisi, A.; Ponti, A., Pebbles and Pebblejuggler: Software for Accurate, Unbiased, and Fast Measurement and Analysis of Nanoparticle Morphology from Transmission Electron Microscopy (TEM) Micrographs. *Nanoscale* 2012, 4, 5356-5372.

38. Guinier, A.; Fournet, G., *Small-Angle Scattering of X-Rays*. John Wiley and Sons: New York, 1955.
39. <http://www.sasview.org>.
40. Finger, L. W.; Hazen, R. M.; Hofmeister, A. M., *High-Pressure Crystal Chemistry of Spinel (MgAl₂O₄) and Magnetite (Fe₃O₄): Comparisons with Silicate Spinel*. *Phys. Chem. Miner.* 1986, 13, 215-220.
41. Gulley-Stahl, H.; Hogan, P. A., 2nd; Schmidt, W. L.; Wall, S. J.; Buhrlage, A.; Bullen, H. A., *Surface Complexation of Catechol to Metal Oxides: An ATR-FTIR, Adsorption, and Dissolution Study*. *Environ. Sci. Technol.* 2010, 44, 4116-4121.
42. Valotteau, C.; Calers, C.; Casale, S.; Berton, J.; Stevens, C. V.; Babonneau, F.; Pradier, C.-M.; Humblot, V.; Baccile, N., *Biocidal Properties of a Glycosylated Surface: Sophorolipids on Au(111)*. *ACS Appl. Mater. Interfaces* 2015, 7, 18086-18095.
43. Slovetiskii, V. I., *Ir Spectra of Nitro Compounds*. *Bull. Acad. Sci. USSR, Div. Chem. Sci.* 1970, 19, 2086-2091.
44. Shirmardi Shaghasemi, B.; Dehghani, E. S.; Benetti, E. M.; Reimhult, E., *Host-Guest Driven Ligand Replacement on Monodisperse Inorganic Nanoparticles*. *Nanoscale* 2017, 9, 8925-8929.
45. Santos-Carballal, D.; Roldan, A.; Grau-Crespo, R.; de Leeuw, N. H., *A Dft Study of the Structures, Stabilities and Redox Behaviour of the Major Surfaces of Magnetite Fe₃O₄*. *Phys. Chem. Chem. Phys.* 2014, 16, 21082-21097.
46. Valotteau, C.; Banat, I. M.; Mitchell, C. A.; Lydon, H.; Marchant, R.; Babonneau, F.; Pradier, C.-M.; Baccile, N.; Humblot, V., *Antibacterial Properties of Sophorolipid-Modified Gold Surfaces against Gram Positive and Gram Negative Pathogens*. *Colloids Surf. B* 2017, 157, 325-334.
47. Chen, M.; Dong, C.; Penfold, J.; Thomas, R. K.; Smyth, T. J. P.; Perfumo, A.; Marchant, R.; Banat, I. M.; Stevenson, P.; Parry, A.; Tucker, I.; Campbell, R. A., *Adsorption of Sophorolipid Biosurfactants on Their Own and Mixed with Sodium Dodecyl Benzene Sulfonate, at the Air/Water Interface*. *Langmuir* 2011, 27, 8854-8866.
48. Baccile, N.; Selmane, M.; Le Griel, P.; Prevost, S.; Perez, J.; Stevens, C. V.; Delbeke, E.; Zibek, S.; Guenther, M.; Soetaert, W.; Van Bogaert, I. N. A.; Roelants, S.,

Ph-Driven Self-Assembly of Acidic Microbial Glycolipids. *Langmuir* 2016, 32, 6343-6359.

49. Abel, S.; Dupradeau, F.-Y.; Raman, E. P.; MacKerell, A. D.; Marchi, M., Molecular Simulations of Dodecyl-B-Maltoside Micelles in Water: Influence of the Headgroup Conformation and Force Field Parameters. *J. Phys. Chem. B* 2011, 115, 487-499.

50. Bergin, R.; Carlstrom, D., Structure of the Pyrocatecholamines. li. Crystal Structure of Dopamine Hydrochloride. *Acta Crystallogr., Sect. B* 1968, 24, 1506-1510.

51. Smith, M. B.; McGillivray, D. J.; Genzer, J.; Loesche, M.; Kilpatrick, P. K., Neutron Reflectometry of Supported Hybrid Bilayers with Inserted Peptide. *Soft Matter* 2010, 6, 862-865.

52. Reimhult, E.; Schroffenegger, M.; Lassenberger, A., Design Principles for Thermoresponsive Core-Shell Nanoparticles – Controlling Thermal Transitions by Brush Morphology. *Langmuir* 2019, 22, 7092-7104

53. Manet, S.; Cuvier, A.-S.; Valotteau, C.; Fadda, G. C.; Perez, J.; Karakas, E.; Abel, S.; Baccile, N., Structure of Bolaamphiphile Sophorolipid Micelles Characterized with SAXS, SANS, and MD Simulations. *J. Phys. Chem. B* 2015, 119, 13113-13133.

54. Penfold, J.; Chen, M.; Thomas, R. K.; Dong, C.; Smyth, T. J.; Perfumo, A.; Marchant, R.; Banat, I. M.; Stevenson, P.; Parry, A.; Tucker, I.; Grillo, I., Solution Self-Assembly of the Sophorolipid Biosurfactant and Its Mixture with Anionic Surfactant Sodium Dodecyl Benzene Sulfonate. *Langmuir* 2011, 27, 8867-8877.

55. Amstad, E.; Gillich, T.; Bilecka, I.; Textor, M.; Reimhult, E., Ultrastable Iron Oxide Nanoparticle Colloidal Suspensions Using Dispersants with Catechol-Derived Anchor Groups. *Nano Letters* 2009, 9, 4042-4048.

56. Zirbs, R.; Lassenberger, A.; Vonderhaid, I.; Kurzhals, S.; Reimhult, E., Melt-Grafting for the Synthesis of Core-Shell Nanoparticles with Ultra-High Dispersant Density. *Nanoscale* 2015, 7, 11216-11225.

57. Ikeda, Y.; Sunakawa, T.; Tsuchiya, S.; Kondo, M.; Okamoto, K., Toxicological Studies on Sophorolipid Derivatives. (I). Acute Toxicity, Eye Irritation, Primary Skin Irritation, Skin Sensitization, Phototoxicity, Photosensitization, Mutagenicity of

Polyoxypropylene (12) [(2'-O-Beta-D-Glucopyranosyl-Beta-D-Glucopyranosyl)Oxy-] Fatty Acid Ester-]. J. toxicol. Sci. 1986, 11, 197-211.

58. Gal, N.; Lassenberger, A.; Herrero-Nogareda, L.; Scheberl, A.; Charwat, V.; Kasper, C.; Reimhult, E., Interaction of Size-Tailored PEGylated Iron Oxide Nanoparticles with Lipid Membranes and Cells. ACS Biomater. Sci. Eng. 2017, 3, 249-259.

59. Varela-Aramburu, S.; Wirth, R.; Lai, C. H.; Orts-Gil, G.; Seeberger, P. H., Straightforward and Robust Synthesis of Monodisperse Surface-Functionalized Gold Nanoclusters. Beilstein J. Nanotech. 2016, 7, 1278-1283.

60. Reynolds, M.; Marradi, M.; Imbert, A.; Penades, S.; Perez, S., Influence of Ligand Presentation Density on the Molecular Recognition of Mannose-Functionalised Glyconanoparticles by Bacterial Lectin Bc2I-A. Glycoconj. J. 2013, 30, 747-757.

61. Ruiz, C. C., Sugar-Based Surfactants; Fundamentals and Applications. [In: Surfactant Sci. Ser., 2009; 143]. CRC Press: 2008.

Graphical Abstract