Micelle carriers based on dendritic macromolecules containing bis-MPA and glycine for antimalarial drug delivery†

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Biomaterials for antimalarial drug transport still need to be investigated in order to attain nanocarriers that can tackle essential issues related to malaria treatment, e.g. complying with size requirements and targeting specificity for their entry into Plasmodium-infected red blood cells (pRBCs), and limiting premature drug elimination or drug resistance evolution. Two types of dendritic macromolecule that can form vehicles suitable for antimalarial drug transport are herein explored. A new hybrid dendritic-linear-dendritic block copolymer based on Pluronic® F127 and amino terminated 2,2′-bis(glycyloxymethyl)propionic acid dendrons with a poly(ester amide) skeleton (HDLDBC-bGMPA) and an amino terminated hyperbranched polymer derived from 2,2′-bis(hydroxymethyl)propionic acid (DHP-bMPA) have provided self-assembled and unimolecular micelles. Both types of micelle carrier are biocompatible and exhibit appropriate sizes to enter into pRBCs. Targeting studies have revealed different behaviors for each nanocarrier that may open new perspectives for antimalarial therapeutic approaches. Whereas DHP-bMPA exhibits a clear targeting specificity for pRBCs, HDLDBC-bGMPA is incorporated by all erythrocytes. It has also been observed that DHP-bMPA and HDLDBC-bGMPA incorporate into human umbilical vein endothelial cells with different subcellular localization, i.e. cytosolic and nuclear, respectively. Drug loading capacity and encapsulation efficiencies for the antimalarial compounds chloroquine, primaquine and quinacrine ranging from 30% to 60% have been determined for both carriers. The resulting drug-loaded nanocarriers have been tested for their capacity to inhibit Plasmodium growth in in vitro and in vivo assays.

Introduction

According to the latest estimates, around 219 million cases of malaria occurred globally in 2017 and the disease led to 435 000 deaths.⁠† People living in the poorest countries are the most vulnerable, with approximately 90% of deaths in Africa, of which 70% are children under 5 years of age. Although increased prevention and control measures have led to a reduction in malaria mortality rates by more than 42% globally since 2000, an estimated 3.4 billion people are still at risk of being infected and developing disease. Despite the undeniable importance of malaria elimination on the global research agenda, current vaccines in development do not offer prospects of complete protection⁠† and the available front-line drugs are rapidly losing efficacy, with resistance already evolved to the first-line drug artemisinin.⁠† As a result, since 2014 the malaria incidence and mortality decline have stalled. Thus, alternative strategies⁠† working through radically new mechanisms are urgently needed. Antimalarial drugs can potentially target a suite of pathogen life stages inside two different hosts: humans and the insect vector. Infection starts when a parasitized female Anopheles mosquito, while taking a blood meal, inoculates sporozoites of the malaria parasite, the
protopist Plasmodium spp. In the liver, sporozoites develop into merozoites, which enter the circulation, invade red blood cells (RBCs) and replicate asexually through ring, trophozoite and schizont stages to produce daughter cells that invade new RBCs to perpetuate the blood-stage cycle. Some parasites eventually differentiate into sexual stages, female or male gametocytes that are ingested by a mosquito from peripheral blood. Following fertilization in the insect’s midgut, the zygote differentiates into an ookinete that moves through the midgut epithelium and forms an oocyst, which releases sporozoites. The malaria transmission cycle is restarted when sporozoites migrate to the salivary glands and are injected into a human with the mosquito’s next bite.

Most chemotherapeutic approaches against malaria are targeted at the asexual, blood-stage parasites that are responsible for all symptoms and pathologies of the disease. Currently administered antimalarial drugs are in free form in the blood circulation and have poor specificity for Plasmodium-infected RBCs (pRBCs). This incurs the risk of having to deliver large overall doses over an extended period of time to compensate for drug removal through spleen and liver clearance and kidney filtration. However, patient non-compliance and low concentration thresholds to ward off potential side effects often end up in sublethal local drug amounts reaching infected cells, which in turn stimulate resistance evolution. Because malaria pathophysiology is so complex and the disease is so widespread, it is generally accepted that to achieve eradication a combination of weapons will be needed. These include the improvement of existing approaches and the development of new ones, with drug therapy remaining the mainstay of treatment and prevention, and nanotechnology being able to provide innovative useful tools. The objective of delivering drugs exclusively to a selected site with minimal exposure for sensitive adjacent healthy cells or tissues is the holy grail of the fast-developing nanomedicine field. Encapsulation of drugs in targeted nanovectors is a rapidly growing area with a clear applicability to infectious disease treatment, and pharmaceutical nanotechnology has been identified as a potentially essential asset to the future fight against malaria.

In the search for suitable nanocarriers for the treatment of infectious diseases, dendrimers stand out as valuable candidates due to their inherent features for building nanostructures with controlled size, morphology and surface functionalization. In a previous work we had explored different amphilic cationic dendritic derivatives based on 2,2′-bis(hydroxyethyl) propionic acid (bis-MPA) as nanocarriers for the targeted delivery of antimalarial drugs. These dendritic derivatives consisted of Janus dendrimers or hybrid dendritic-linear-dendritic block copolymers (HDLDBC), and both self-assembled into micelles with glycine groups at the surface. Although some of these structures exhibited specific pRBC targeting and in vitro antimalarial activity, they had an excessive unspecific toxicity, a very large size after antimalarial drug loading (>150 nm), and their in vivo tests had been preliminary.

Here we build on our previous work to explore new specifically targeted delivery systems for antimalarial drugs of a size after drug encapsulation (<30 nm) sufficiently small to facilitate their entry into pRBCs. Accordingly, two types of cationic dendritic derivative are proposed, which mainly differ in the way they form the nanocarrier, either self-assembled micelles or unimolecular micelles. As for the former, and on the basis of the promising results shown by the previously reported Pluronic® F127 HDLDBC derivative that contained polyester dendrons, we have herein conjugated Pluronic® F127 with poly(ester amide) dendrons to generate a new micelle-forming self-assembling amphiphilic cationic HDLDBC (Fig. 1A). The combination of the hydrolytic degradability of ester linkages and the stability and H-bond-forming ability of amide groups has proven very interesting for the design of synthetic polymers for biomedical applications. Accordingly, a poly(ester amide) dendron conjugated at both ends of Pluronic® F127 has been herein envisaged as a possibility to modulate the size and stability of self-assembled micelles given the additional H-bonding interactions due to amide groups. The dendron employed derives from the monomer 2,2′-bis(glyclyoxyethyl)propionic acid (bis-GMPA), which was recently described by us as one of the few examples of poly(ester amide) dendritic structures reported in the literature, which showed high potential for biomedical applications due to the biocompatibility, degradability and positive results in drug and gene delivery of its dendritic derivatives. As for the achievement of a unimolecular micelle carrier, we have

![Fig. 1 Chemical structure and cartoon representation of (A) micelle formation by HDLDBC-bGMPA, a hybrid dendritic-linear-dendritic block copolymer composed by Pluronic F127 and dendrons derived from 2,2′-bis(glyclyoxyethyl)propionic acid (bis-GMPA), and (B) DHP-bMPA, dendronized hyperbranched polymers derived from 2,2′-bis(hydroxyethyl) propionic acid (bis-MPA) hyperbranched polymers and bis-MPA dendrons functionalized with glycine groups.](image-url)
selected dendronized hyperbranched polymers (DHPs), also
called pseudo-dendrimers, based on a hyperbranched polymer
of bis-MPA, conjugated at its periphery with bis-MPA dendrons
with exposed glycine moieties (Fig. 1B). We demonstrated that
these DHP-bMPA derivatives could deliver DNA into mesenchy-
mal cells. However, their potential as drug carriers remains
to be explored. In this respect, these polymers showed appro-
priate biocompatibility and degradability that make them
interesting to be investigated as carriers for antimalarial drug
delivery.

Both types of structure have been investigated for their tar-
geting towards pRBCs, and the drug loading capacity and
encapsulation efficiencies of the carriers have been tested with
the antimalarial compounds chloroquine (CQ), primaquine
(PQ) and quinacrine (QN). The resulting nanocarriers have
been assayed for their capacity to inhibit Plasmodium growth
in in vitro and in vivo assays.

Materials and methods
Reagents
Unless otherwise indicated, all reagents were purchased from
Sigma-Aldrich® or Acros,™ and used always without further
purification. Dichloromethane (DCM) and tetrahydrofuran
(THF) were dried using solvent purification systems.

Synthesis and characterization of the dendritic derivatives
Globular DHP-bMPA dendronized hyperbranched polymers as
well as DHP-bMPA-Rho, a DHP containing covalently linked
rhodamine B fluorophore, were synthesized as previously
reported by us. HDLDBC-bGMPA was prepared by copper
azide–alkyne cycloaddition (CuAAC) coupling of the t-Boc pro-
tected bis-GMPA dendron and the Pluronic® F127 bis(alkyne)
derivative as depicted in Fig. 2A.

Fig. 2 Synthesis of HDLDBC-bGMPA (A) and chemical characterization by 1H NMR (B), 13C NMR (C), FTIR (D) and SEC (E). 1H NMR spectra with
signal relative integration, the 1H–1H COSY NMR spectra and full 13C NMR spectra of HDLDBC-bGMPA are gathered in the ESI.
**Synthetic procedure and analytical data of t-Boc protected HDLDBC-bGMPA.** Alkylene functionalized Pluronic® F127 (1.00 g, 7.74 × 10^{-2} mmol, 1.00 eq.) and bis-GMPA dendron (NHBOc)2 (514 mg, 2.01 × 10^{-2} mmol, 2.60 eq.) were dissolved into 8 mL of dimethylformamide (DMF) in a Schlenk flask and 3 vacuum–argon cycles were made to remove the air. The reaction mixture was stirred under argon atmosphere at 45 °C. CuSO4·5H2O (18.2 mg, 6.19 × 10^{-3} mmol, 0.80 eq.) was dissolved into DMF (4 mL) in a second Schlenk flask and exposed to 3 vacuum cycles; then the copper solution was added through a cannula to the previous mixture of DCM and distilled water (1 : 1) at feeding ratios of 1 : 1, and a saturated solution of HCl(g) (7 mL) in ethyl acetate was carefully added to it. The reaction was stirred at room temperature for 45 min until a white gel appeared. The mixture was dialyzed against methanol for 24 h to obtain a light yellow solid (855 mg, 61%).

1H NMR (400 MHz, CDCl3) δ (ppm): 1.11 (m, 201H), 1.27 (s, 6H), 1.32 (s, 20H), 1.39 (s, 24H), 1.64 (m, 4H), 1.95 (m, 4H), 3.47 (m, 67H), 3.51–3.73 (m, ~1000H), 3.84 (t, δ = 6.8 Hz, 4H), 4.00 (s, 8H), 4.03 (s, 32H), 4.12 (t, δ = 6.0 Hz, 4H), 4.31 (m, 24H), 4.45 (m, 40H) 5.30 (s, 4H), 7.38 (d, δ = 8.8 Hz, 4H), 8.23 (d, δ = 8.8 Hz, 4H), 8.19 (s, 2H). 13C NMR (125 MHz, CD2OD) δ (ppm): 17.4–18.3, 26.3, 26.9, 29.3, 31.1, 41.5, 42.2, 47.3, 51.3, 62.6–76.8, 115.9, 124.2, 125.8, 132.8, 140.4, 163.7, 167.6, 168.8, 170.8–171.2, 174.0, 174.7–175.0. FTIR (κmax/cm⁻¹): 3600–3300 (bs N–O st carbamate), 1545 (N–O st amide), 1468 (CH2–, CH3 δ), 1099 (C–O–C at st).

**Characterization techniques.** 1H and 13C Nuclear Magnetic Resonance (NMR) experiments were performed using a Bruker AV-400 (1H: 400 MHz, 13C: 100 MHz) or a Bruker AV-500 (1H: 500 MHz, 13C: 125 MHz) spectrometer (Bruker Corporation, Billerica, MA, USA), employing as solvents deuterated chloroform (CDCl3), deuterated methanol (CD2OD) or deuterated dimethyl sulfoxide ((CD3)2SO). The chemical shifts are indicated in ppm relative to tetramethylsilane (TMS) and the coupling constant in Hz; the solvent residual peak was used as internal standard for spectrum calibration. Fourier transformed infrared (FTIR) spectroscopy was performed using a Bruker Vertex 70 spectrophotometer in ATR (attenuated total reflectance) mode and recorded between 4000 and 800 cm⁻¹.

Size exclusion chromatography (SEC) was performed with a Waters e2695 Alliance employing two in series HR4 and HR1 Styragel columns (500 and 104 Å of pore size, respectively) and a Waters 2424 evaporation light scattering detector with a sample concentration of 1 mg mL⁻¹ in THF (HPLC grade) with a flow rate of 1 mL min⁻¹ at 35 °C; poly(methyl methacrylate), PMMA, was used as standard for calibration.

**Self-assembly of HDLDBC-bGMPA.** HDLDBC-bGMPA was mixed with the corresponding amount of distilled water and cooled down to 4 °C for 30 min until complete dissolution. Then, the solution was slowly heated to room temperature to trigger the formation of the water soluble nanocarriers. The critical micelle concentration (CMC) was determined using the Nile Red technique. Briefly, 2 mL of distilled water solutions of HDLDBC-bGMPA with concentrations ranging from 0.1 to 5 mg mL⁻¹ were prepared at 25 °C. Nile Red was dissolved in ethanol at a concentration of 0.25 mM and 10 µL of this solution was added to each sample. The mixtures were then stirred at room temperature for 1 h in the dark with an orbital shaker. The fluorescence emission spectrum of each solution was recorded with a PerkinElmer LS 55 fluorimeter after excitation at λex = 550 nm.

**Preparation of carrier-antimalarial drug conjugates and drug release assays**

The antimalarial compounds CQ, PQ and QN were encapsulated within the corresponding carrier (DHP-bMPA or HDLDBC-bGMPA) following the oil-in-water procedure previously employed by us for CQ and PQ. Briefly, each dendritic derivative and the corresponding drug were dissolved into a mixture of DCM and distilled water (1 : 1) at feeding ratios of wDHP : wdrug = 1 : 0.5 and wHDLDBC : wdrug = 1 : 1. The samples were vigorously stirred at room temperature employing an orbital shaker under ventilation until complete evaporation of DCM (around 2 h). Non-encapsulated drug was removed by
dialysis ( regenerated cellulose membrane, MW 1000 Da cut off, Spectra/Port®) against distilled water (200 mL) at 4 °C for 16 h. The amount of encapsulated drug was indirectly determined: the quantity of drug present in the dialysis water was measured by UV-VIS spectrometry (Varian Cary50 Probe UV-visible spectrophotometer) at the wavelengths of $\lambda_{\text{A(CQ)}} = 345$ nm for CQ, $\lambda_{\text{A(PQ)}} = 259$ nm for PQ and $\lambda_{\text{A(QN)}} = 280$ nm for QN and was subtracted from the initially incorporated drug. The samples were freeze-dried with a Telstar Cryodos 50 freeze-dryer in order to increase their stability over long storage periods.

The release of CQ encapsulated within HDLDBC-bGMPA and DHP-bMPA was studied by dialysis against phosphate buffered saline (PBS). 2 mL of the dendrimer/drug conjugates were dialyzed against 200 mL of PBS (regenerated cellulose dialysis membrane, MW 1000 Da cut-off, Spectra/Port®) under stirring at 37 °C. For CQ determination, aliquots (2 mL) were withdrawn at different times from the waters of dialysis up to 72 h (specifically at 0, 2, 4, 6, 24, 48, and 72 h). An identical procedure was performed with a control solution of free CQ at the same concentration as in the conjugate preparation.

**Fluorescent labeling of HDLDBC-bGMPA**

HDLDBC-bGMPA nanocarriers were labeled by encapsulating a low water soluble modified rhodamine B (Rho(C17)2) red fluorophore previously reported by us,27 and the oil-in-water procedure was employed to encapsulate the fluorophore within the nanocarrier. The selected feeding ratio in this case was (1:0.15) ($w_{\text{HDLDBC}}:w_{\text{Rho}}$). The release profile of Rho(C17)2 from the dendrimer/Rho(C17)2 conjugates was studied by dialysis similarly as the drug release procedure described above. In this case, 2 mL of the HDLDBC-bGMPA/Rho(C17)2 conjugate containing 0.15 mg mL$^{-1}$ of encapsulated Rho(C17)2 were dialyzed (regenerated cellulose membrane, MW 2000 Da cut off, Spectra/Port®) against distilled water (200 mL) at 37 °C. At different times up to 72 h, 2 mL aliquots were withdrawn from where the quantity of Rho(C17)2 was determined by measuring fluorescence intensity ($\lambda_{\text{ex}} = 540$ nm, $\lambda_{\text{em}} = 580$ nm).

**Transmission electron microscopy (TEM) and atomic force microscopy (AFM) analysis**

TEM images were obtained with a FEI TECNAI T20 electron microscope (FEI Company, Eindhoven, The Netherlands) with 200 kV beam power, using holey carbon film 300 mesh coppered grids (Agar Scientific Ltd). A droplet of an aqueous solution of the sample at a concentration of 1 mg mL$^{-1}$ was deposited on the grid and left for 30 s, removing the excess aqueous solution by blotting with filter paper. A droplet of a 3% w/v aqueous solution of phosphotungstic acid used as negative stain was deposited on the grid and left for 10 s before removing the excess staining solution by blotting with filter paper. The grid was dried for at least 24 h under atmospheric pressure at room temperature. The average size of the different nanocarriers was obtained by analysing $\geq$100 structures in $\geq$4 TEM images.

For AFM analyses, 10 µL of 1 µg mL$^{-1}$ or 10 ng mL$^{-1}$ dendrimer solution in double deionised water (ddH$_2$O; MilliQ system, Millipore) were deposited on cleaved mica substrates and, after an adsorption time of about 5 min, 40 µL ddH$_2$O were added. High-resolution images were obtained with a MultiMode 8 atomic force microscope equipped with a NanoScope V controller (Bruker Corporation) operating in ScanAsyst mode in liquid, using ScanAsyst-Fluid + probes (Bruker Corporation).

**Plasmodium falciparum cell culture and parasite growth inhibition assay**

*P. falciparum* 3D7 was grown *in vitro* in human RBCs of blood group type B prepared as described elsewhere by using previously established conditions.29 Briefly, parasites (thawed from glycerol stocks) were cultured at 37 °C in T25 flasks (SPL Life Sciences) containing RBCs in Roswell Park Memorial Institute (RPMI) complete medium (supplemented with 5 g L$^{-1}$ Albumax II and 2 mM glutamine) under a gas mixture of 92% N$_2$, 5% CO$_2$, and 3% O$_2$. Synchronized ring stage cultures were obtained by 5% sorbitol lysis and synchronized late stage cultures were obtained using 70% Percoll® (GE Healthcare) purification to enrich in late trophozoites and early schizonts;30 the medium was changed every 2 days maintaining 3% hematocrit. For culture maintenance, parasitemias were kept below 5% late forms by dilution with fresh RBCs. The human blood used in this work was commercially obtained from the Banc de Sang i Teixits (http://www.bancsang.net). Blood was not specifically collected for this research; the purchased units had been discarded for transfusion, usually because of an excess of blood relative to anticoagulant solution. Prior to their use, blood units underwent the analytical checks specified in the current legislation. Before being delivered to us, unit data were anonymized and irreversibly dissociated, and any identification tag or label had been removed in order to guarantee the non-identification of the blood donor. No blood data were or will be supplied, in accordance with the current Ley Orgánica de Protección de Datos and Ley de Investigación Biomédica. The blood samples will not be used for studies other than those made explicit in this research. Experiments were approved by the Ethics Committee of the Hospital Clínico de Barcelona.

To isolate *P. falciparum* merozoites, a 3D7 strain culture was tightly synchronized (sorbitol lysis on day 1, 70% Percoll® followed after 2 h by sorbitol on day 4, sorbitol on day 6), and after a further 40 h a final 70% Percoll® treatment was done. Purified late stages were cultured in 10 mL of complete RPMI without adding fresh RBCs, and when the majority of parasites were segmented schizonts, E-64 protease inhibitor was added to a final concentration of 10 µM. Between 6 to 10 h later, the culture was centrifuged at 730 g for 8 min and the pelleted cells were taken up in 20 mL of PBS. The suspension was passed sequentially through a 18G needle and a 1.2 µm filter (Sartorius Stedim® Minisart) blocked for 30 min with PBS/1% BSA. Merozoites in PBS were collected in a 50 mL Falcon tube previously blocked for 1 h with PBS/1% BSA, and finally centri-
to 75 µL. After addition of 0.5 µM Syto-11 (Thermo Fisher Scientific, Inc.), parasitemia was determined by flow cytometry as previously described.\(^{28}\)

### Cytotoxicity and hemolysis assays

Human umbilical vein endothelial cells (HUVEC, ATCC) were cultured in Medium 199 (M199, LabClinics) supplemented with 10% heat-inactivated foetal bovine serum (FBS, PAA Laboratories, Germany), 1% each penicillin/streptomycin (Biological Industries), and 10 mM glutamine (complete M199). 5000 cells per well were plated in 96-well plates (Thermo Fisher Scientific Inc.) and after 24 h at 37 °C in 5% CO\(_2\) atmosphere the medium was substituted by dilutions of the dendrimers in 100 µL of culture medium without FBS, and incubation was resumed for 48 h. 10 µL of 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate labeling reagent (WST-1, Roche Diagnostics GmbH) was added to each well, and the plate was incubated in the same conditions for a further 3 h. After thoroughly mixing for 1 min on a shaker, the absorbance of the samples was measured at 541 nm.

### Determination of the maximum tolerated dose (MTD) in mice

Seven week-old inbred BALB/cAnR mouse females (18–20 g, Janvier Laboratories) were maintained under standard environmental conditions (20–24 °C and 12 h/12 h light/dark cycle) with ad libitum access to food and water. To ensure administration and minimize injection stress, the animals were anesthetized with isoflurane (4% for induction and 2.5% for maintenance) in an oxygen stream, while a 250 µL bolus was administered intravenously. To reduce the number of animals used, an adaptation of OECD 425 Test Guideline was followed, which consisted of a single ordered dose progression. The first mouse received the dose initially planned for antimalarial activity assays (see below), and the dose for the next animal was either increased or decreased by a factor of 3.2 depending on whether the first animal survived or died, respectively. Each mouse was injected and evaluated for at least 48 h before the next animal was treated. All animals were observed for toxic signs during 14 days after dose injection. Following this protocol, three different concentrations of the dendrimers (11.7, 37.5 and 120 mg kg\(^{-1}\)) were evaluated, prepared in PBS from a 50 mg mL\(^{-1}\) stock solution of dendrimers in sterile ddH\(_2\)O. In the presence of toxic effects including, among others, >20% reduction in animal weight, aggressive and unexpected animal behavior or the presence of blood in faeces, animals were immediately anesthetized using a 100 mg kg\(^{-1}\) Ketolar plus 5 mg kg\(^{-1}\) Midazolan mixture and sacrificed by cervical dislocation. Dendrimer MTD was therefore defined as the highest dosage exhibiting an absence of the aforesaid toxicity signs. The animal care and use protocols followed adhered to the specific national and international guidelines.
Results and discussion

Preparation and characterization of the nanocarriers

Synthesis and characterization of HDLDBC-bGMPA. HDLDBC-bGMPA was synthesized for the first time for this study by CuAAC of the bis(alkyne) derivative of Pluronic® F127 and two t-Boc protected amino-terminated bis-GMPA dendrons (Fig. 2A and ESI†). The t-Boc-protected bis-GMPA dendron of 3rd generation with an azide group at the focal point and eight terminal amino groups was synthesized as previously described by us.24 The synthesis of the alkyne functionalized Pluronic® was carried out by esterification of Pluronic® F127 with 4-(prop-2-ynyloxy)benzoic acid as we reported previously.18 The CuAAC reaction was performed in DMF at 45 °C under argon atmosphere during 2 days. The Cu(i) catalytic species was formed “in situ” by reduction of Cu(II) salt with (l)-ascorbate while TBTA was added to increase its stability. An excess of dendron (1.3 mole of dendron per 1.0 mole of alkyne group) was used to obtain a complete functionalization of the linear polymer. The t-Boc protecting groups were removed from the dendrons in acidic conditions with HCl in ethyl acetate and the final product was obtained with a moderate yield of 61%.

The correct grafting of the two bis-GMPA dendrons at the extremities of Pluronic® F-127 was first controlled by 1H (Fig. 2B) and 13C (Fig. 2C) NMR experiments (see Fig. S1.1, S1.2 and S1.3† for 1H-1H COSY and full 1H and 13C NMR spectra). The appearance of a peak at 8.19 ppm (H-15) in the 1H NMR spectrum and two peaks at 125.8 (C-15) and 140.4 ppm (C-14) in the 13C NMR spectrum confirmed the formation of the triazole rings. Additionally, three other signals were shifted downfield in the 1H NMR spectra of the final HDLDBC when compared with the spectra of its two precursors. Thus, the signal corresponding to the methylene protons in the α-position of the triazole rings belonging to the linear polymeric part (H-13) was shifted from 4.72 to 5.30 ppm. The signals corresponding to the methylene protons in the α- and β-positions of the triazole rings belonging to the dendritic part (H-16 and H-17) were shifted from 3.20 and 1.65 to 4.45 and 1.95 ppm, respectively. Moreover, the relative integrations of the signals corresponding to the dendrons and linear polymer corroborated the grafting of two dendrons at the terminal position of the Pluronic® F-127 (Table S1.1†).

The characteristic bands of the bis-GMPA dendrons and Pluronic® F-127 could be observed in the FTIR spectrum, (Fig. 2D). First, the presence of the poly(ether) Pluronic® F-127 was asserted by the intense band at 1101 cm⁻¹ (C–O bond vibration stretching) and the two intense bands at 2881 and 1344 cm⁻¹ (C–H bond vibrations). Second, the presence of the poly(ester amide) bis-GMPA dendrons was confirmed by the two bands at 1755 and 1664 cm⁻¹ corresponding to C=O bond vibration stretching of ester and amide groups, respectively.

SEC was performed with the t-Boc-protected precursor of HDLDBC-bGMPA due to its low solubility in the elution solvent employed for chromatography, i.e. THF (Fig. 2E). Two
peaks were observed in the chromatograms of both, the commercial Pluronic® F-127 and the HDLDBC, showing the polydispersity of these compounds. The correct functionalization of Pluronic® was nevertheless asserted as both peaks corresponding to it showed lower retention time than the two peaks corresponding to the starting Pluronic® F-127. Additionally, no peak corresponding to residual free bis-GMPA dendron could be observed. In summary, all experimental data obtained during the characterization of HDLDBC-bGMPA confirmed the correct insertion of two bis-GMPA dendrons at each extremity of the linear polymer and the absence of unreacted free linear polymer or dendron.

**Structural studies.** The ability of HDLDBC-bGMPA to self-assemble in water forming micellar nanocarriers was confirmed by CMC determination using the Nile Red method (Fig. 3A). The fluorescence intensity of this solvatochromic fluorophore increases drastically when it migrates into and is retained within the lipophilic part of aggregates formed by the self-assembly of amphiphilic molecules. Thus, a CMC of 1.0 mg mL\(^{-1}\) for HDLDBC-bGMPA was determined, which was consistent with the formation of micelles in which the lipophilic core of the central linear Pluronic® block copolymer is surrounded by hydrophilic ammonium terminated dendrons. Rounded structures were observed in AFM and negatively stained TEM images of HDLDBC-bGMPA. The average diameter calculated from TEM micrographs was 13 ± 3 nm, consistent with the height of the structures determined in water by AFM (Fig. 3B and C), and with the average in number value measured by DLS, 26 ± 6 nm (Fig. S2.1.A†).

The morphology of two globular dendronized hyperbranched polymers, DHP-bMPA, was also analyzed by TEM and AFM (Fig. 3D–F). The two generations tested (\(n = 3\) and \(n = 4\) in Fig. 1B) appeared as rounded objects, with average diameters calculated from TEM images of 9.8 ± 2.7 nm and 13.5 ± 3.5 nm, respectively. These dimensions are consistent with the expected formation by these dendronized hyperbranched polymers of unimolecular micelles well dispersed in aqueous solution. DLS measurements of DHP-bMPA (\(n = 4\)) gave a diameter of 11 ± 2 nm (Fig. S2.1.B†). As for HDLDBC-bGMPA carriers, the size of DHP-bMPA unimolecular micelles results a priori appropriate to enter into pRBCs.

**Unspecific toxicity and hemolysis assays.** None of the dendritic derivatives was cytotoxic or hemolytic up to a concentration of 0.15 mg mL\(^{-1}\) (Fig. 3G and H), which is 10 times higher than the highest amount to be used later for in vitro growth inhibition assays, and 8 times higher than the daily dose to be used later for in vivo assays.

### In vitro targeting analysis

**Rhodamine labeling of the nanocarriers.** In order to explore the targeting behavior of the nanocarriers towards pRBCs, DHP-bMPA-Rho (Fig. 4A), a DHP-bMPA covalently labeled with an average of seven rhodamine B moieties per macromolecule, was prepared as previously described. HDLDBC-bGMPA nanocarriers were labeled by encapsulating a lipophilic modified rhodamine B (Rho(C17)\(_2\)) red fluorophore, using the oil-in-water procedure as previously reported by us. In order to check the stability of this labeling, Rho(C17)\(_2\), release from the HDLDBC-bGMPA/Rho(C17)\(_2\) conjugates was studied by dialysis. Unmodified water-soluble rhodamine B fluorophore was encapsulated within HDLDBC-bGMPA micelles following the same protocol, to be used as a control. The resulting release profiles showed that whereas 50% of the encapsulated rhodamine B is released during the first 2 h, Rho(C17)\(_2\) is kept encapsulated throughout the 72 h of the experiment (Fig. 4B).

**Erythrocyte targeting analysis.** Fluorescence microscopy analysis of non-fixed samples (Fig. 5A) showed that whereas HDLDBC-bGMPA was incorporated by all RBCs, DHP-bMPA exhibited at the same concentration a clear targeting specificity for pRBCs. Higher-resolution confocal fluorescence microscopy images of DHP-bMPA-containing samples revealed that the polymer fluorescence was associated to the pRBC plasma membrane and to intraerythrocytic parasites (Fig. 5B). In the latter case fluorescence was not detected in the RBC cytosol and was circumscribed by the plasma membrane of *Plasmodium*. These observations suggested a specific binding of DHP-bMPA to *Plasmodium falciparum* antigens.
The differential RBC targeting of DHP-bMPA and HDLDBC-bGMPA was confirmed by flow cytometry assays (Fig. 6); whereas HDLDBC-bGMPA associated with *P. falciparum* merozoites and with both parasitized and non-parasitized erythrocytes, DHP-bMPA interacted only with merozoites and with pRBCs. HDLDBC-bGMPA interaction with both RBCs and pRBCs does not invalidate it as a potential carrier of antimalarial drugs in targeted delivery strategies. As we have shown before, delivering antimalarial compounds to non-infected erythrocytes might represent an interesting therapeutic approach whereby *Plasmodium* would encounter a hostile environment since the very first moment after RBC inva-
sion.\textsuperscript{35,36} This scenario resulted in a significantly improved efficacy of drugs encapsulated inside liposomes targeted to both pRBCs and to non-parasitized red blood cells.\textsuperscript{35,37}

The modest binding of DHP-bMPA to early ring forms, where the parasite has not significantly modified the erythrocyte membrane, suggests that this polymer might interact predominantly with exported \textit{Plasmodium} antigens, which are scarce in rings and completely absent from non-parasitized RBCs.

**Endothelial cell targeting analysis.** Other cell types besides RBCs will be exposed to targeted polymers used as drug carriers in the blood stream, mainly leukocytes and blood vessel endothelium. Of these, leukocytes are in numbers several orders of magnitude below those of RBCs, but endothelial cells are sufficiently abundant to compete with pRBCs for nanocarrier intake. We have shown above that, at the polymer concentrations used in this work, endothelial cells do not experience significant cytotoxic effects. However, they might contribute to the clearance from the blood of a significant amount of polymers, thus reducing the efficacy of nanovector preparations. When endothelial cell uptake of the polymers was analyzed by fluorescence microscopy, it was observed that both DHP-bMPA and HDLDBC-bGMPA were incorporated by HUVEC cells. However, whereas HDLDBC-bGMPA had a cytosolic localization, the subcellular targeting of DHP-bMPA was found to be exclusively nuclear (Fig. 7). This result offers interesting perspectives for the use of this type of polymers in the targeting to cell types other than erythrocytes and to different subcellular locations. Nevertheless, the observed interaction of both polymers with the endothelial lining has to be taken into account in future pharmacokinetic studies regarding potential applications as drug carriers against the blood stages of malaria.

**In vitro antimalarial activity of drug-loaded nanocarriers**

**Drug encapsulation.** CQ, PQ and QN were encapsulated within DHP-bMPA and HDLDBC-bGMPA nanocarriers. Except where otherwise indicated, the DHP-bMPA structure used hereafter in this work will be $n = 4$. The encapsulation was performed following the oil-in-water method at feeding ratios of $1:0.5$ (wDHP/wdrug) and $1:1$ (wHDLDBC/wdrug), respectively. This procedure allowed to encapsulate all three antimalarial drugs in DHP-bMPA nanocarriers with drug loading contents around 20\% in weight and good encapsulation efficiencies, ranging from 37\% to 60\% (Table 1). A smaller DHP-bMPA ($n = 3$ in Fig. 1B) was also used to encapsulate all three antimalarials, obtaining similar drug contents and loading efficiencies (Table S2.1). As for the HDLDBC-bGMPA carrier, higher loading capacities, between 30\% and 48\% in weight, and rather good encapsulation efficiencies, ranging from 31\% to 48\%, were attained.

All the aqueous solutions obtained after the encapsulation procedure were freeze-dried in order to enhance stability during storage of the carrier/drug systems. The effective encapsulation of drugs was asserted by comparing the absorption spectra of the drugs in water with those of the re-dissolved carrier/drug freeze-dried conjugates (Fig. S2.3 and S2.4). In all cases, the differences observed reflect interactions between nanocarriers and drugs.

TEM images of drug-containing nanocarriers showed in all cases homogeneous dispersions of rounded objects (Fig. 8), the diameters of which were similar to those of empty nanocarriers, and always $\leq$20 nm (Table 1). This small size should favor their entry in pRBCs, since \textit{Plasmodium} induces new permeation pathways that confer to the host cell an increased permeability to a wide range of particles up to diameters of 50–70 nm.\textsuperscript{31} Whereas the unimolecular micelles formed by DHP-bMPA in water (Fig. 8A) had an average diameter around 13.5 nm, DHP-bMPA/drug systems had an average diameter between 12 and 20 nm. Likewise, the loading of antimalarial drugs within HDLDBC-bGMPA nanocarriers resulted in structures with average sizes ranging from 11 to 17 nm (Fig. 8B), whereas the empty nanocarrier had an average size around 13 nm. AFM observation of both CQ-loaded carriers confirmed the formation of these rounded objects (Fig. 8).

**In vitro antimalarial activity assays.** When the corresponding formulations of HDLDBC-bGMPA and DHP-bMPA

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**Table 1** Encapsulation of CQ, PQ and QN in HDLDBC-bGMPA and DHP-bMPA nanocarriers and average diameters of the carrier/drug systems determined by TEM

<table>
<thead>
<tr>
<th>Drug</th>
<th>mg drug/mg carrier</th>
<th>EE\textsuperscript{a} (%)</th>
<th>Average diameter (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HDLDBC-bGMPA</td>
<td>Empty</td>
<td>0.307</td>
<td>31</td>
</tr>
<tr>
<td></td>
<td>PQ</td>
<td>0.408</td>
<td>41</td>
</tr>
<tr>
<td></td>
<td>QN</td>
<td>0.475</td>
<td>48</td>
</tr>
<tr>
<td>DHP-bMPA</td>
<td>Empty</td>
<td>0.246</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>PQ</td>
<td>0.214</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>QN</td>
<td>0.229</td>
<td>37</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Encapsulation efficiency (EE, \%) represents the fraction of encapsulated drug relative to the quantity used to perform the encapsulation.

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**Fig. 7** Fluorescence microscopy cell targeting analysis of rhodamine-labeled DHP-bMPA and HDLDBC-bGMPA to HUVEC cells.
conjugated with CQ, PQ and QN were tested in growth inhibition assays of *P. falciparum* in vitro cultures, the encapsulated drugs had IC$_{50}$ values comparable to those of the free compounds (Fig. 9), indicating an adequate release of the drugs from the polymers. However, the good targeting of the nanostructures towards pRBCs offered prospects for a better performance of the encapsulated drugs, suggesting that part of the nanoparticle-conjugated compounds might not be adequately released. To explore this possibility, the release dynamics against PBS of CQ encapsulated within HDLDBC-bGMPA and DHP-bMPA was analyzed during three days (Fig. 10). As already described for other dendritic derivatives,$^{18}$ CQ was progressively released from both nanocarriers in a process that reached completion in about 24 h; after this time, about 35% of CQ remained stably encapsulated. Modifications of the polymer architecture or chemistry to endow it with more porosity or less affinity for the drugs might contribute to an improved drug release profile.

**In vivo antimalarial activity of CQ-loaded nanocarriers**

MTD assays indicated that DHP-bMPA and HDLDBC-bGMPA could be safely administered to mice without evident signs of animal distress up to respective doses of at least 37.5 and 120 mg kg$^{-1}$. For the evaluation of the formulations in an *in vivo* model of malaria, we selected CQ because, being the *in vitro* performance of its encapsulated form vs. the free compound similar to that of PQ and QN, this drug had been suc-
cessfully tested in our group as part of different polymeric and liposomal encapsulations.\textsuperscript{18,35,38} When polymer-CQ conjugates were administered intravenously (1.9 mg CQ per kg per day for 4 consecutive days) to mice infected with the lethal murine malaria parasite \textit{P. yoelii yoelii} \textit{17XL (PyL) MRA-267}, DHP-bMPA-CQ did not improve survival significantly, but animals treated with HDLDBC-bGMPA-CQ survived much longer (3 out of 5 mice lived \textgreater 20 days) than untreated controls, which typically died at day 6 (Fig. 11A and C). One mouse treated with HDLDBC-bGMPA-CQ was actually cured and, when re-infected at day 69 and left untreated, it recovered completely without showing any symptoms of malaria or developing parasitemia above the detection level of microscopy examination. The detection in the plasma of this cured animal of antibodies against \textit{P. yoelii} antigens (Fig. 11B) was consistent with the development of immunity against the disease. Free CQ-treated controls which were administered the same drug concentration were cured in 4 out of 5 animals, which became also resistant to re-infection. Although HDLDBC-bGMPA-encapsulated CQ did not improve the efficacy of such a good drug as CQ, the strategy presented here can be adapted to the targeted drug delivery of other antimalarials, already existing or yet to be discovered.

\textit{In vivo}, HDLDBC-bGMPA-CQ worked better than DHP-bMPA-CQ, possibly because the former targets all RBCs. The loading of antimalarial drugs into non-parasitized red blood cells has been described as an efficient approach to significantly reduce parasite survival\textsuperscript{35,36} as long as neither nanoparticles nor drugs affect the natural role of erythrocytes as \textit{O}_{2} and \textit{CO}_{2} transporters. Both dendrimeric structures studied in this work lack significant \textit{in vitro} cytotoxicity and hemolytic activity, suggesting that they will not interfere with the red blood cell physiology. HDLDBC-bGMPA then holds promise for the development of innovative antimalarial prophylactic strategies at the cell level whereby \textit{Plasmodium} would be exposed to drugs since the very first moment after invading a host cell.

The modest \textit{in vivo} efficacy of the encapsulated formulations relative to the free drug is, in part, likely resulting from renal and splenic clearance of the nanoparticles, in addition to their endothelial cell uptake. Nanoparticles must be larger than 20 nm in diameter to avoid filtration by the kidney,\textsuperscript{39} and smaller than 100 nm to avoid a specific sequestration by sinusoids in spleen and fenestra of liver, which are approximately 150–200 nm in diameter.\textsuperscript{40} Therefore, systemically administered nanoparticles should have diameters from 20 to 100 nm,\textsuperscript{41} and overall, literature suggests that nanoparticles in the 50–100 nm size range display the lowest blood clearance rates.\textsuperscript{42} Since the average diameter of the dendrimeric nanoparticles used here is just below 20 nm, increasing their size to ca. 50 nm might provide an improved pharmacokinetics and better \textit{in vivo} performance.

In addition, upon intravenous administration of nanoparticles, these are coated by a variety of serum proteins which are recognized by the scavenger receptor on macrophage cell surfaces and internalized, leading to a significant loss of nanoparticles from the circulation.\textsuperscript{43} The serum proteins binding on the nanoparticles are also termed “opsonins”, and the macrophages contributing the major loss of injected dose are also known as the reticuloendothelial system or mononuclear phagocyte system. Reducing protein binding is the key point for developing a long-circulation nanoparticle formulation. To minimize opsonization, the most commonly used strategy is to conjugate onto the surface of the nanoparticles the polyethylene glycol polymer\textsuperscript{44} or polyoxazolines,\textsuperscript{45} both hydrophilic polymers that provide good steric hindrance for preventing protein binding.

Finally, in addition to expanding blood residence time and reducing unspecific interactions with non-target cells and tissues, a faster targeting dynamics can be conferred to the nanoparticles by coating them with molecules binding pRBCs with certain degree of specificity, as it has been described for heparin.\textsuperscript{46,47} Here we have shown that although DHP-bMPA exhibited a remarkable preferential binding to pRBCs vs. RBCs, about 40% of pRBCs interacted with this polymer similarly as a fraction of the non-infected erythrocyte population did (Fig. 6). The functionalization of DHP-bMPA-Rho with up to 15% heparin-FITC (w/w) did not affect the targeting specificity of the polymer towards pRBCs (Fig. S3.1†), and it actually favored specific interactions with pRBCs vs. RBCs (data not shown). The known activity of
heparin as targeting element of liposomes suggests that its presence on the nanocarrier increased the number of polymers bound to each pRBC. Last but not least, since heparin has antimalarial activity, this result indicates that it can be a constituent of pRBC-targeted multicomponent nanoparticles carrying different types of antimalarial agents.

Conclusions

To eradicate malaria, there is an urgent need for the combination of different strategies working together, because it is unlikely for a single approach to be capable of success. It is important to emphasize the importance of implementing in advance efficient methods of antimalarial drug encapsulation and targeted delivery in order to make a good use of future therapeutic compounds. This should result in a more rational administration regime that could contribute to expand the time during which drugs can be used before resistance evolves. In addition, such strategy might widen the number of chemicals that reach the clinic if eventual unspecific toxicities can be reduced through a targeted delivery approach. The resulting lower overall doses should not trigger pernicious side-effects in the patient while keeping high local doses on the parasite in order to quickly eliminate all the cells of the pathogen, thus reducing the evolution of resistances. Here, we have described micellar carriers based on dendritic macromolecules containing bis-MPA and gly cine that hold promise for the development of future antimalarial nanomedicines targeted to both Plasmodium-infected and non-infected erythrocytes.

Authors contributions

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

Conflicts of interest

There are no conflicts to declare.

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Notes and references