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Amphiphilic dendritic derivatives as nanocarriers for the targeted delivery of antimalarial drugs

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ABSTRACT

It can be foreseen that in a future scenario of malaria eradication, a varied armamentarium will be required, including strategies for the targeted administration of antimalarial compounds. The development of nanovectors capable of encapsulating drugs and of delivering them to *Plasmodium*-infected cells with high specificity and efficacy and at an affordable cost is of particular interest. With this objective, dendritic derivatives based on 2,2-*bis*(hydroxymethyl)propionic acid (*bis*-MPA) and Pluronic® polymers have been herein explored. Four different dendritic derivatives have been tested for their capacity to encapsulate the antimalarial drugs chloroquine (CQ) and primaquine (PQ), their specific targeting to *Plasmodium*-infected red blood cells (pRBCs), and their antimalarial activity *in vitro* against the human pathogen *Plasmodium falciparum* and *in vivo* against the rodent malaria species *Plasmodium yoelii*. The results obtained have allowed the identification of two dendritic derivatives exhibiting specific targeting to pRBCs vs. non-infected RBCs, which reduce the *in vitro* IC_{50} of CQ and PQ by ca. 3- and 4-fold down to 4.0 nM and 1.1 μM, respectively. This work on the application of dendritic derivatives to antimalarial targeted drug delivery opens the way for the use of this new type of chemicals in future malaria eradication programs.

1. Introduction

Because the blood-stage infection is responsible for all symptoms and pathologies of malaria, *Plasmodium*-infected red blood cells (pRBCs) are a main chemotherapeutic target [1]. However, the success of antimalarial therapies is significantly reduced due to a variety of factors mostly derived from the complexity of the parasite life cycle and the emergence of drug resistance [2]. Consequently, new antimalarial drugs with ever increasing potency are being developed [3], many of them with a narrow therapeutic window. Drug delivery strategies could play an important role in the treatment of malaria because they might allow (i) low overall doses to limit the toxicity of the drug for the patient, (ii) delivery of sufficiently high local amounts to avoid the development of resistant parasite strains [4,5], (iii) improvement of the efficacy of currently used hydrophilic (low membrane trespassing capacity) and lipophilic antimalarials (poor aqueous solubility), (iv) use of orphan drugs never assayed as malaria therapy, e.g. because of their high and unspecific toxicity, and (v) increased immune responses in vaccine formulations.

Malaria parasites have evolved resistance (first reported from the field between 1 and 15 years after introduction, depending on the drug) to all classes of antimalarials that have gone into widespread use [6]. The physiopathology of *Plasmodium* has mechanisms oriented to develop such resistance [7], which suggests that most future new drugs will follow the same fate of rapidly losing efficacy. A strategy to maintain for a longer time the activity of yet to be discovered antimalarials is to design in advance new biomaterials for administration methods allowing the highly targeted delivery of drugs to infected cells. The development of novel drug

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delivery systems is not only less expensive than finding new drugs, but may also improve release of antimalarials at the desired rates [8]. Nanomedicine, which uses nanosized tools for the treatment of disease [9], has not been extensively applied to malaria yet, but the administration of antimalarial compounds would largely benefit from a method based on nanoparticles able to deliver their encapsulated drugs into pRBCs with high specificity. Nanoparticulate systems are a miscellaneous family of submicron structures, typically self-assembling and unable to self-replicate, and the main feature that makes them attractive drug carriers is their small size, up to several hundred nm, which allows them to cross biological barriers. Furthermore, nanoaggregates play a protective role for the drugs that helps to increase their circulating half lifes and water solubility, thus improving their therapeutic efficacy [10].

Polymer-based nanoaggregates are among the most promising carriers for drug delivery applications [11–13]. Recently, polyamidoamine (PAA)-derived polymers have been studied for the administration of antimalarials to pRBCs [14]. The PAAs AGMA1 and ISA23 exhibited specific binding and entry into target cells, but their *in vitro* IC_{50} improvement of encapsulated drugs was modest. This, in addition to their relatively small drug encapsulation capacity led us to explore new polymeric structures that could offer an alternative to PAAs. In particular, dendrimers and dendrons form a especial type of monodisperse polymers synthesized through generational growth [15–17], which have shown great potential for the design of efficient vehicles for drug delivery, mainly forming either covalent or guest-host drug-dendrimer conjugates [18–23]. The possibility of precisely tailoring the structure of dendritic molecules makes them excellent candidates to design self-assembling units that form nanoaggregates in solution [24–27], which in turn could trap molecules. Amphiphilic dendrimers (including dendrimers and dendritic polymers) have been described to form a variety of supramolecular nanostructures [28–30], enabling the effective internalization of their encapsulated drugs into cells [31].

On the basis of these considerations, we have addressed the development of efficient antimalarial carriers towards the design of two types of dendritic systems susceptible of self-aggregating in water while encapsulating the drug: amphiphilic segmented dendrimers, also called Janus dendrimers [28,30,32], consisting of two dendritic blocks of different polarity, and hybrid dendritic-linear-dendritic block copolymers [33–35] formed by a linear amphiphilic polymer functionalized at both ends with dendritic blocks. In the search of advantageous dendritic building blocks for the amphiphilic systems, we focused our attention on polyester dendrimers based on 2,2-*bis*(hydroxymethyl)propionic acid (*bis*-MPA) monomers [36]. Hult and colleagues first described this type of structures in 1996 [37] and their application in the biomedical field has been developed since then [38–42]. Some characteristics of *bis*-MPA derivatives, among which their biocompatibility *in vitro* and *in vivo*, their solubility in biological environment, their ability to be degraded by enzymes or by nonenzymatic hydrolysis and their ease of functionalization, make them good candidates for drug delivery.

2. Materials and methods

2.1. Reagents

Unless otherwise indicated, all reagents were purchased from Sigma–Aldrich (St. Louis, MO, USA) and used as received.

2.2. Synthesis and characterization of the dendritic derivatives A, B, C and D

The experimental details concerning the synthesis and characterization of all final and intermediate compounds synthesized are included in the Supplementary Information. Janus dendrimers A and B (Fig. 1) were synthesized by copper-catalyzed 1,3-dipolar azide-alkyne cycloaddition [43] of the azido-terminated glycine containing dendron and the alkyne-terminated stearic acid-functionalized dendron. A and B were obtained with total yields of 16% and 3%, respectively. Hybrid dendritic-linear-dendritic block copolymers C and D (Fig. 1) were prepared from the commercial amphiphilic block copolymer Pluronic® F127. Compound C was obtained in three steps, starting with the synthesis of the bis-MPA anhydride. The anhydride reacted directly with the terminal hydroxyl groups of the polymer to give a derivative with a first-generation bis-MPA dendron at each end of the polymeric chain. Finally, the hydroxyl terminal groups of the bis-MPA moieties were functionalized with glycine giving rise to the final compound C with a total yield of 53%. Compound D was also obtained in three steps. During the first step, the terminal hydroxyl groups of Pluronic® F127 were esterified with 4-(prop-2-ynyloxy)benzoic acid. In a second step, the alkyne terminal groups were coupled via 1,3-dipolar cycloaddition with an azido-terminated dendron derived from the third generation bis-MPA, which had the terminal hydroxyl groups protected with Boc-glycine groups (See the synthesis of compound B-3a in the Supplementary Information). Finally, the deprotection of the amine groups gave compound D in a total yield of 38%. The compounds A, B, C and D, as well as the intermediates, were characterized by ^{1}H NMR, ^{13}C NMR, FTIR, mass spectroscopy and elemental analysis (details in Supplementary Information).

2.3. Encapsulation of drugs and rhodamine B and release assays

The oil/water method described by Vrignaud et al. [12], based on the emulsification of an organic phase including the corresponding dendritic derivative (compound A, B, C or D) and an aqueous phase including the drug, chloroquine (CQ) or primaquine (PQ), or rhodamine B was used to form the nanovectors (Fig. 2). Chloroquine diphosphate, primaquine diphosphate and rhodamine B were solubilized in water and the polymer was added in a small amount of dichloromethane at a molar ratio drug:polymer 5:1. After 1 h stirring and the complete evaporation of dichloromethane, the sample was dialyzed (24 h, 4 °C) against double deionized water (MilliQ system, Millipore) in order to remove free drug and free rhodamine B (cellulose membrane, 2000 Da cutoff, Spectrum® Laboratories). These nanoparticles could be stored frozen up to several months. The amount of encapsulated compounds was calculated in an indirect way by subtracting their content in the dialysis water, measuring the absorbance at 345 nm or 340 nm for chloroquine and primaquine, respectively, and the fluorescence emission of rhodamine B at 580 nm. The encapsulation efficiency (EE) is expressed as mole of encapsulated compound/mole of initially added compound × 100.

For release assays, dendritic derivatives conjugated to chloroquine, primaquine, or rhodamine B were diluted in 250 μL of Roswell Park Memorial Institute (RPMI) complete medium supplemented with 0.5% Albumax and dialyzed (Slide-A-Lyzer MINI Dialysis Device, 10K MWCO, 0.1 mL, Thermo Scientific) at room temperature against 5 mL of the same medium for up to 48 h. 100-μL samples were taken at the specified times from the waters of dialysis and placed in a 96-well plate for determination of the different compounds as specified above.

2.4. Scanning electron microscopy (SEM)

For SEM analyses, 20 μL of the suspension of nanovectors in water was deposited on a glass plate and the solvent was evaporated at room temperature. Gold coating was done with an SC7620 Mini Sputter Coater (Quorum Technologies), and the samples were imaged with an Inspect TM550 SEM (FEI Company). The average aspect ratio (AR) was calculated for the different nanovectors following the formula AR = length/width, with 1 corresponding to a perfect sphere. The structures have been considered spherical when $1 < AR \leq 1.2$, ovoid when $1.2 < AR \leq 3$, and elongated when $AR > 3$.

2.5. Plasmodium falciparum cell culture and growth inhibition assays (GIAs)

Plasmodium falciparum 3D7 was grown *in vitro* in rinsed human RBCs of blood group type B prepared as described elsewhere [44] using previously established conditions [45]. Briefly, parasites (thawed from glycerol stocks) were cultured at 37 °C in Petri dishes containing RBCs in RPMI complete medium under a gas mixture of 92% N_2, 5% CO_2, and 3% O_2. Synchronized cultures were obtained by 5% sorbitol lysis [46], and the medium was changed every 2 days maintaining 3% hematocrit. For culture maintenance, parasitemias were kept below 5% late forms by dilution with washed RBCs. For GIAs, parasitemia was adjusted to 1.5% with more than 90% of parasites at ring stage after sorbitol synchronization. 200 μL of these living *Plasmodium* cultures were plated in 96-well plates and incubated for 48 h at 37 °C in the presence of free drugs, polymers, or polymer-encapsulated drugs. Parasitemia was determined by microscopic counting of blood smears or by fluorescence-assisted cell sorting as previously described [44].

2.6. Confocal fluorescence microscopy

Living *P. falciparum* cultures with mature stages of the parasite were incubated in phosphate buffered saline (PBS) in the presence of 100 μg/mL of polymers encapsulating rhodamine B for 90 min at 37 °C with gentle stirring. After washing, blood smears were prepared and cells were fixed in acetone:methanol (90:10). Parasite nuclei were stained with 4′,6-diamino-2-phenylindole (DAPI, Invitrogen) and the RBC membrane was labeled with wheat germ agglutinin (WGA)-Alexa 488