

Giant Host Red Blood Cell Membrane Mimicking Polymersomes Bind Parasite Proteins and Malaria Parasites

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[§]SCS-Metrohm Award for best oral presentation in Polymers, Colloids and Interfaces

Abstract: Malaria is an infectious disease that needs to be addressed using innovative approaches to counteract spread of drug resistance and to establish or optimize vaccination strategies. With our approach, we aim for a dual action with drug- and ‘vaccine-like’ activity against malaria. By inhibiting entry of malaria parasites into host red blood cells (RBCs) – using polymer vesicle-based (polymersome) nanomimics of RBC membranes – the life cycle of the parasite is interrupted and the exposed parasites are accessible to the host immune system. Here, we describe how host cell-sized RBC membrane mimics, formed with the same block copolymers as nanomimics, also bind the corresponding malaria parasite ligand and whole malaria parasites, similar to nanomimics. This was demonstrated using fluorescence imaging techniques and confirms the suitability of giant polymersomes (GUVs) as simple mimics for RBC membranes.

Keywords: Giant polymersomes · GUV · Malaria · Membrane mimics · *Plasmodium*

Introduction

Block copolymer-based vesicles, called polymersomes^[1] due to their increased mechanical stability compared to liposomes and broad chemical versatility to introduce *e.g.* stimuli-responsiveness – are considered ideal candidates for drug delivery purposes, as nanoreactors, as artificial organelles inside cells, or as simple cell mimics.^[2,3] Our recently developed nanomimic strategy against infectious diseases is based on such polymer vesicles imitating a host cell membrane at the nanoscale (nanomimics), which was achieved by mixing two distinct block copolymers: vesicle-forming poly(2-methyl-2-oxazoline)-*block*-poly(dimethylsiloxane)-*block*-poly(2-methyl-2-oxazoline) (PMOXA-*b*-PDMS-*b*-PMOXA), and PDMS-*b*-heparin.^[4,5] Giant polymersomes (giant unilamellar vesicles, GUVs), several micrometers in diameter, are increasingly acknowledged as interesting model systems of very simplified cell mimics.^[6]

Malaria, a disease caused by *Plasmodium* spp. parasites that are transmitted by *Anopheles* mosquitoes – killing more than 600'000 people each year^[7] – is our current target. When reaching the blood stage cycle, the malaria parasite form called merozoite attaches to and enters red blood cells (RBCs), where the parasite asexually divides to form more parasites, which egress from the infected RBC (iRBC) after about 48 hours and infect more RBCs.^[8] Heparan sulphate (very similar to heparin) on RBCs is considered as being responsible for the initial attachment of merozoite to the RBC by interaction with *Plasmodium falciparum* merozoite surface protein 1-42 (*PfMSP1*₄₂).^[9] With our approach we block the malaria blood stage life cycle using RBC-membrane-mimicking polymersomes, which block the parasite after egress from iRBCs, inhibit invasion, and expose the infective merozoites to the immune system. If successful *in vivo*, this artificial inhibition of merozoite invasion could lead to an immune boost against extracellular merozoites.^[4]

Here, we demonstrate that RBC membrane mimics formed at a larger size, but with similar hollow sphere architecture (heparin GUVs), can also bind the parasite protein *PfMSP1*₄₂ and the whole *Plasmodium* parasite (merozoite form) in a similar fashion as their nano-sized counterparts, the nanomimics.^[4,5] This indicates that GUVs indeed represent a suitable model of small polymersomes,

with the advantage of a size close to that of RBCs. Certain aspects, *e.g.* binding of *PfMSP1*₄₂ to polymersomes can be studied with GUVs using methods that would not be suitable for the nanostructure (*e.g.* confocal laser scanning microscopy (CLSM) to study protein binding).

Experimental

Materials

PDMS-*b*-heparin, PMOXA-*b*-PDMS-*b*-PMOXA, and *PfMSP1*₄₂-OG488 synthesis and characterization can be found in our previous publications.^[4,5]

Giant Polymersome Formation

Giant polymersome (GUV) formation was accomplished according to the standard electroformation technique^[10] using a Nanion Vesicle Prep Pro setup (Nanion Technologies, Munich, Germany). A freshly cleaned ITO-coated glass slide was first plasma-treated (Plasma Cleaner, PDC-002, Harrick Plasma, Ithaca, New York, USA), and then, a thin polymer film was deposited on it. For control GUVs (only PMOXA-*b*-PDMS-*b*-PMOXA) a solution of PMOXA-*b*-PDMS-*b*-PMOXA in ethanol (6 mg/mL, 40 μ L) was dispersed on the ITO-coated side of the glass slide and ethanol was subsequently evaporated using a vacuum chamber for 30 min (Plasma Cleaner, PDC-002, Harrick Plasma, Ithaca, New York, USA). For the heparin GUVs a mixture of PMOXA-*b*-PDMS-

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b-PMOXA in ethanol (6 mg/mL, 40 μ L) and PDMS-*b*-heparin in ethanol (4 mg/mL, 10 μ L) corresponding to a ratio of wt/wt 85:15 was used. Afterwards, a greased O-ring was put around the polymer film on the glass slide to form a chamber and then an aqueous sucrose solution (300 mM, 600 μ L) was added to hydrate the polymer film. After covering by another ITO-coated glass slide, the sample was exposed to an ac current (3.0 V, 3.0 Hz) at room temperature. For control GUVs we performed electroformation overnight and for heparin GUVs electroformation was conducted for one hour. Finally, the GUV solutions were transferred to Eppendorf tubes and stored at 4 $^{\circ}$ C.

Fluorescence Microscopy

Fluorescence imaging of protein interaction with giant polymersomes was performed on a confocal laser scanning microscope (Zeiss CLSM 510-META/Confocor2, Carl Zeiss, Jena, Germany). A freshly plasma-cleaned 8-well microscopy chamber (Nunc Lab-Tek Chamber Slide System, Thermo Fisher Scientific) was filled with PBS (200 μ L, pH 7.2), GUVs (20 μ L in 300 mM sucrose), Bodipy630 (10 μ L, 72 μ M) in PBS, and *Pf*MSP1₄₂-OG488 in PBS (5 μ L, 2.5 μ M). For the CLSM measurements a water immersion objective (C-Apochromat, 40 x magnification, NA of 1.2) was used. The OG488 signal was obtained by excitation with an Ar laser ($\lambda = 488$ nm) with dichroic mirrors HFT (main beam splitter) 488 and NFT (secondary beam splitter) 490 with the band-pass BP 500-550 filter. HFT 488/543/633, NFT 545 and the long-pass LP 650 filters were used to collect the fluorescence signal of Bodipy630. The pinhole size was set to 70 μ m for the green channel, and 90 μ m for the red channel, respectively. All settings (including laser power, detector gains) were kept constant for imaging of positive and negative samples.

Fluorescence imaging of heparin GUVs with merozoites was performed on a Leica DM 5000B fluorescence microscope using the appropriate filters.

Study Interaction of Giants and Merozoites

Plasmodium falciparum 3D7 parasite culturing and merozoite isolation was performed as described elsewhere.^[4,11] Briefly, a 30 mL petri dish filled with human RBCs at 5% haematocrit and 5–6% parasitemia (iRBC late stages) was used for merozoite isolation. First, late stage iRBCs were separated from RBCs and young iRBCs by Percoll density gradient. After maturation to schizont stage parasites, they were concentrated to 1 mL in malaria culture medium, and viable merozoites were mechanically released using 1.2 μ m syringe

filters. They were immediately mixed with 300 μ L of a heparin GUV solution in 300 mM sucrose, gently shaken, and incubated under static conditions at 37 $^{\circ}$ C for 15 min. Subsequently, 2 μ L of 1 mg/mL Hoechst (DNA stain) was added and merozoite/giant mixture was fixed in 2% paraformaldehyde/0.2% glutaraldehyde in 150 mM phosphate buffer (pH 7.4) overnight. After gentle centrifugation (3500 RCF) for 5 min, 10 μ L of the pellet was mounted on a microscopy slide.

Results and Discussion

Recently, we demonstrated that polymersome-based nanomimics of host RBC membranes efficiently block malaria parasite invasion into host RBCs *in vitro*; nanomimics were more than two orders of magnitude more potent invasion inhibitors compared to soluble receptor-like molecules (heparin), when IC₅₀ values were compared based on the heparin concentration in the assays.^[4,5] Furthermore, we measured that several heparin chains on a single nanomimic bound parasite ligands *Pf*MSP1₄₂-OG488 with a high affinity (K_d of 12.1 ± 1.6 nM) using fluorescence cross-correlation spectroscopy (FCCS) of green labelled ligand and red labelled nanomimics.^[5] This multiple binding with high affinity is the basis for a strong multivalent interaction of nanomimic and merozoite, explaining the potent invasion inhibition by nanomimics.^[5]

Herein, host RBC membrane-mimick-

ing polymersomes were formed at a larger size (micrometer scale, heparin GUVs) to study malaria protein and whole parasite interaction with RBC-sized polymersome-based mimics (Fig. 1). The same two block copolymers previously used for nanomimic formation were also used to form these giant mimics. PDMS₆₅-*b*-heparin₁₂ was mixed with PMOXA₅-*b*-PDMS₅₈-*b*-PMOXA₅ (or PMOXA₉-*b*-PDMS₆₇-*b*-PMOXA₉) using 15 wt% of the first block copolymer and subsequent electroformation, yielding heparin GUVs. GUVs made from PMOXA₅-*b*-PDMS₅₈-*b*-PMOXA₅ without the heparin-functionalized copolymer were used as controls.

Giant Host Red Blood Cell Membrane Mimicking Polymersomes Bind Plasmodium Proteins

Formation of GUVs and heparin GUVs was successfully achieved in both cases using electroformation. Usually, a large number of mainly unilamellar, univesicular polymersomes were formed, whereas only few giants with multivesicular structures were found. Interestingly, these artificial and flexible membranes possess a thickness of about 11 nm,^[4] whilst all the membrane components can laterally diffuse within the membrane,^[12,13] but still form stable vesicles with several tens of micrometers in diameter. We performed CLSM to study the interaction of fluorescently labelled *Plasmodium* ligand *Pf*MSP1₄₂-OG488 and heparin, the receptor-like molecule known to bind *Pf*MSP1₄₂,^[9] on the surface

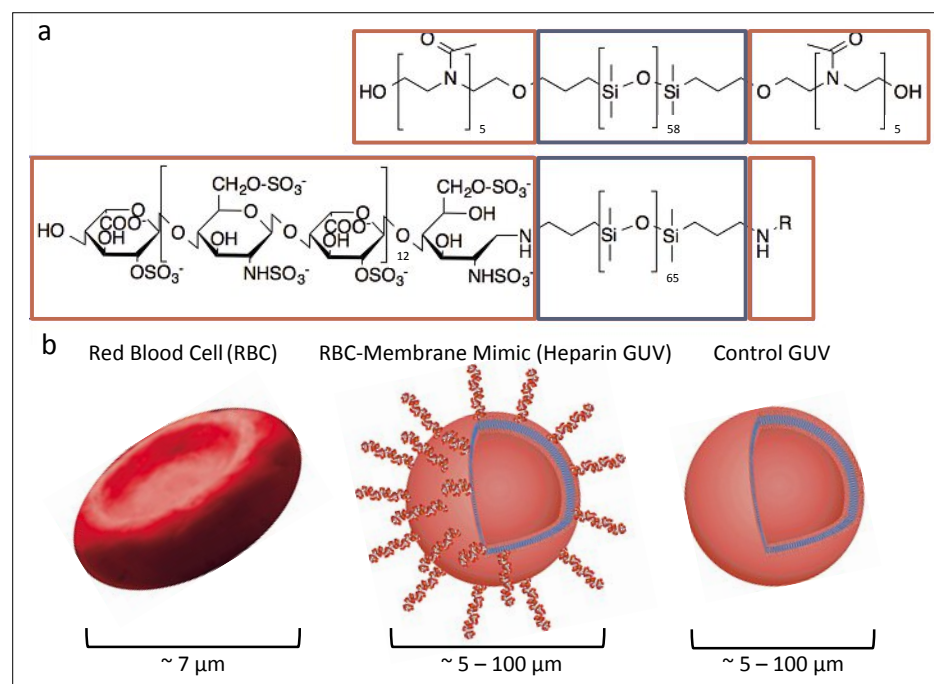


Fig. 1. a) Representation of the chemical structure of the two used block copolymers: PMOXA₅-*b*-PDMS₅₈-*b*-PMOXA₅ and PDMS₆₅-*b*-heparin₁₂. R is either another heparin chain or hydrogen. b) Schematic representation of a red blood cell (RBC), a RBC-membrane-mimetic giant polymer-some (heparin GUV), and a control GUV without the heparin-containing copolymer.

of heparin GUVs. This is only possible with GUVs and not with the nanomimics, because of resolution limitations. The membrane of control GUVs and heparin GUVs were labelled with the hydrophobic dye Bodipy630 for visualizing all the membranes in the red channel (Fig. 2). The hydrophobic dye spontaneously incorporates into the hydrophobic part of the membranes (PDMS).

The receptor-ligand interaction was followed after mixing control GUVs and heparin GUVs with green-labelled *PfMSP1*₄₂-OG488, respectively. In the control experiments, where GUVs without the receptor-like polysaccharide were used, no significant interaction with the parasite protein was observed as expected (Fig. 2c,d). Only the heparin GUVs, which mimic the host RBC membrane, were covered with *PfMSP1*₄₂-OG488 proteins on the surface, which appeared as green rings, whilst the exact same imaging settings as in the experiments with GUVs were used (Fig. 2a,b). Therefore, we could successfully observe the interaction of heparin on GUVs with the *Plasmodium* ligands *PfMSP1*₄₂ using conventional CLSM imaging. This confirms measurements performed using nanomimics and fluorescence-based diffusion measurements (fluorescence correlation spectroscopy (FCS), and FCCS) with the same *PfMSP1*₄₂-OG488 ligands of merozoites.^[4,5] This indicates that the membrane-bound receptor-like molecules (heparin) were also exposed on the large heparin GUVs after electroformation, similarly to the nano-scaled versions thereof (nanomimics). Therefore, GUVs are well suited to study certain biophysical aspects of the interaction between the parasite proteins and membrane mimics with a size relevant biologically, which support the conclusions already reported for the corresponding nanoversion system (nanomimics).^[4,5]

Giant Host Red Blood Cell Membrane Mimicking Polymersomes Bind Plasmodium Merozoites

Successful binding of parasite proteins to heparin GUVs encouraged us to perform experiments with whole *Plasmodium* merozoites to check for binding of viable parasites to heparin GUVs. This was studied using fluorescence microscopy after incubation of heparin GUVs with purified, viable *Plasmodium falciparum* merozoites, which is the most aggressive, life-threatening species of malaria parasites. The membrane of heparin GUVs was again visualized by red, hydrophobic dye (Bodipy630). Fluorescence images clearly demonstrate that merozoites also bind to RBC-sized host membrane-mimetic polymersomes (Fig. 3). In both examples shown, the

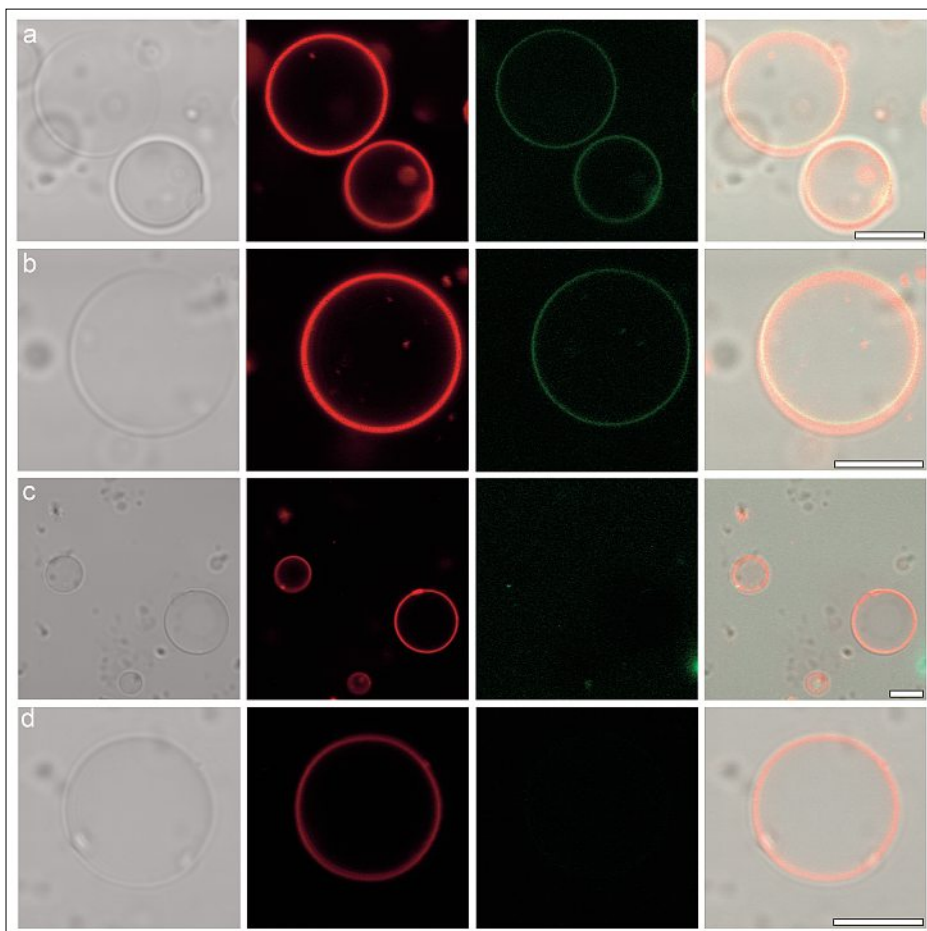


Fig. 2. CLSM images of heparin GUVs (a, b) and control GUVs (c, d), which were mixed with the *Plasmodium* parasite ligand *PfMSP1*₄₂-OG488 to investigate the ligand-receptor interaction; only when the receptor was presented on the surface (heparin GUVs), the parasite ligand bound to the GUVs, control GUVs showed no non-specific interaction with the protein. Left: DIC image. Middle left: Bodipy630 in the hydrophobic part of the GUV membranes. Middle right: OG488 on *PfMSP1*₄₂. Right: Merge. Scale bars: 10 μm .

merozoites slightly indented the membrane of heparin GUVs upon binding. This deformation of the polymersome structure upon merozoite binding was also seen with nanomimics as previously demonstrated by electron microscopy of ultrathin slices of merozoite-nanomimic complexes.^[4]

This confirms that large-scaled poly-

mer vesicles can mimic – at a very primitive stage – the membrane of the host RBC to such an extent that binding of *Plasmodium* proteins and whole malaria parasites is achieved. The large size of GUVs allows the analysis of certain aspects by conventional imaging methods. Naturally, for a real *in vivo* application the nano-scaled

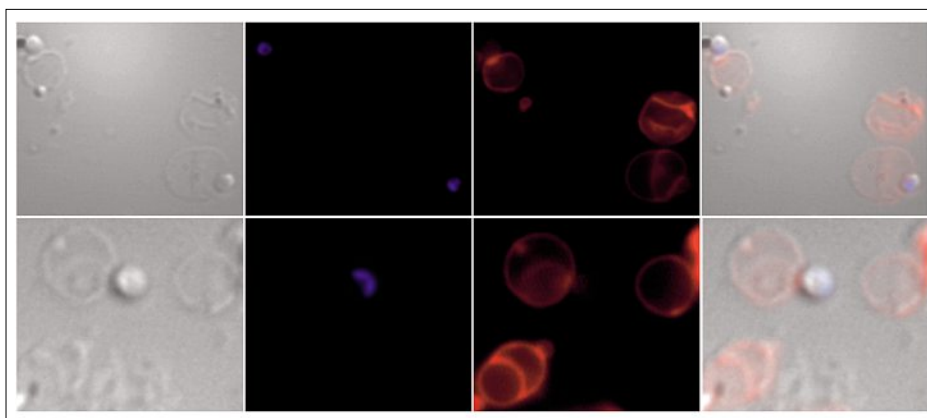


Fig. 3. Fluorescence microscopy images of giant host RBC-mimicking polymersomes (red) that bound *Plasmodium* merozoites (blue, DNA stain). Left: DIC image. Middle left: Hoechst, DNA stain. Middle right: Bodipy630, staining polymersome membranes. Right: Merge. Merozoites are about 1.5 μm long.

versions are preferable, but certain aspects are difficult to analyze using only nano-sized structures.

Conclusion

Host RBC-membrane-mimicking polymersomes (heparin GUVs) were successfully formed at a corresponding RBC size (about 7 μm disk diameter) using two block copolymers and electroformation technique. Only when the second copolymer, containing the receptor-like molecule (heparin), was present in the GUV membrane, the parasite ligand PfMSP1₄₂ bound to the GUVs. Furthermore, whole, viable, and RBC-infecting *Plasmodium falciparum* merozoites interacted and deformed these large-sized RBC membrane mimics (heparin GUVs). These are important steps towards understanding and de-

fining this concept and to advance closer to a real biomedical application.

Acknowledgements

The Swiss National Science Foundation, NCCR 'Molecular Systems Engineering', and the University of Basel are acknowledged for financial support. A.N. gratefully acknowledges Metrohm Schweiz AG and the Swiss Chemical Society for the award. Authors thank S. Kasper for synthesizing PMOXA-*b*-PDMS-*b*-PMOXA and E. Angov (Division of Malaria Vaccine Development, Walter Reed Army Institute of Research, Silver Spring, MD, USA) is kindly acknowledged for providing PfMSP1₄₂.

Received: December 27, 2015

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