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Research Note

Functional Polymeric Microspheres for the Detection of Anti-NANP Antibodies in Falciparum Malaria Patients

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Previously, the presence of the antibodies to sporozoite in the sera of individuals was determined by using the whole sporozoite from dissected mosquitoes as the antigen. A series of successive studies on *Plasmodium falciparum* sporozoite antigen have presented evidence that the immunodominant region of the circumsporozoite (CS) protein is a peptide that contains twelve amino acids consisting of three repeats of L-asparaginyl-L-alanyl-L-asparaginyl-L-proline [$(NANP)_3$] sequence (Zavala *et al.*, 1983; Dame *et al.*, 1984). Determination of the antigen structure offers a way to use a synthetic peptides as a better immunodiagnostic tool (Zavala *et al.*, 1986; Campbell *et al.*, 1987), overcoming the difficulties associated with large scale serologic studies. Synthetic peptides consisting of three or more repeats of the NANP unit inhibited the binding of the monoclonal antibodies to extracts of sporozoites, while the $(NANP)_2$ peptides failed to do so (Zavala *et al.*, 1985). It has been speculated that the synthetic peptide consisting of larger amount of aminoacids is more likely to adopt the conformation of the native protein. By comparing the antibody responses to the intact CS and to the synthetic peptide, it was concluded that the $(NANP)_3$ peptide can be used in immunoassays (Brown *et al.*, 1989) to confirm recent sporozoites invasion from infected anopheles mosquitoes. However, in the present study, we synthesized a methacryloyl monomer conjugated peptide consisting of a single NANP unit only, and further produced polymeric microspheres with the NANP units in the side chain after polymerization of this monomer. Potentiality of this microsphere as an antigenic material detecting anti-sporozoite antibody is discussed.

Microspheres for an immunoassay were synthesized in the following way. Briefly; t-butoxycarbonyl-protected-L-asparaginyl-L-alanyl-L-asparaginyl-L-proline methyl ester (Boc-NANPOMe) was first synthesized by conventional solution phase synthetic method. After removal of the Boc residue, the methacryloyl-activated tetrapeptide (MA-NANPOMe) was then synthesized by acylation of methacryloylchloride. The product was purified by recrystallization from tetrahydrofuran-hexane solution, and its structure (Fig. 1) was identified by Nuclear Magnetic Resonance and Fourier Transform Infrared Spectroscopy measurements. The solutions of MA-NANPOMe and diethylene glycol dimethacrylate (2G) in propionic acid were deoxygenated by nitrogen bubbling and irradiated at a ^{60}Co γ source at room temperature without stirring. The irradiation dose was in the range of 5 to 30 kGy, at a constant dose rate of 10 kGy/h. After irradiation, the produced microspheres were washed with the

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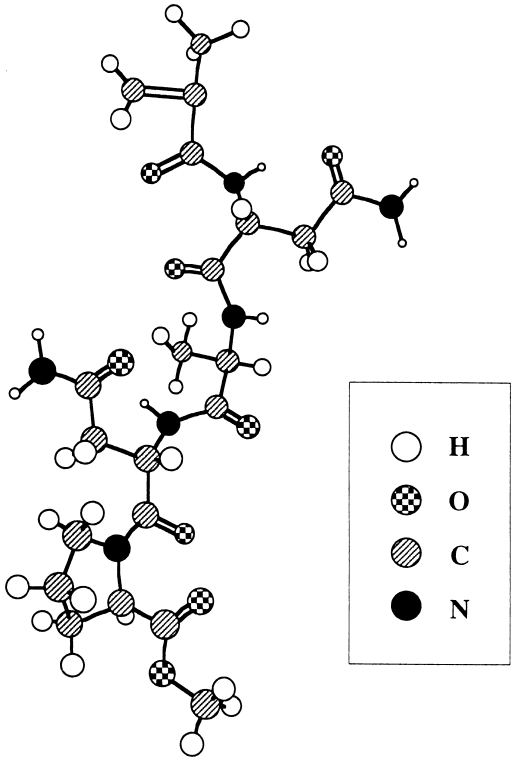

Fig. 1 Illustration of the chemical structure of the MA-NANPOMe monomer.

solvent, then with ethanol and water, centrifuged and freeze-dried. Thus, we obtained a spherical polymer with the numerous NANP units in the side chain (Fig. 2).

To test the assumption that the NANP units on the microspheres can be recognized by the antibodies directed against the CS protein, we employed the indirect fluorescent antibody assay (IFA) using sera from ten symptomatic and parasitemic falciparum malaria patients. One was an inhabitant in an endemic area in the Philippines and the others were imported malaria patients who developed falciparum malaria after coming back to Japan. All the patients were considered to have developed parasitemia within a short time after having been bitten by infected mosquitoes. As a negative control, 10 sera from healthy Japanese donors, who had never contracted malaria, were used. The IFA was done in the following way: MA-NANPOMe microspheres were suspended in patients' sera of different dilutions, for 1 h at 37°C, washed, and reacted with ×100 diluted fluorescein isothiocyanate conjugated rabbit anti-human IgG (DAKO, Denmark) for 30 min at 37°C. After washing, the microspheres were observed by the confocal laser scanning microscope (BioRad,

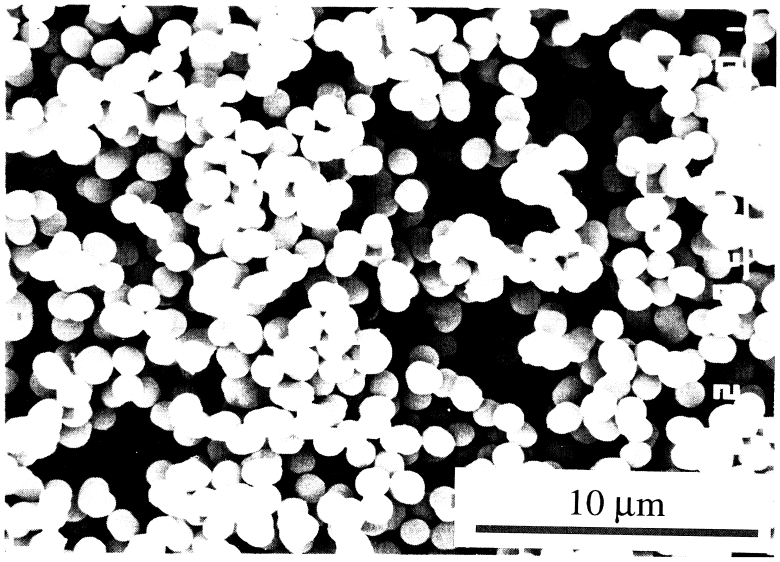

Fig. 2 A scanning electron micrograph of poly(MA-NANPOMe-2G) microspheres of 1 μm in diameter, taken at magnification of 4000. The spheres were obtained from a 10%w/v co-monomer solution with 1:9 molar ratio of MA-NANPOMe to 2G. The solution was irradiated with 10kGy.

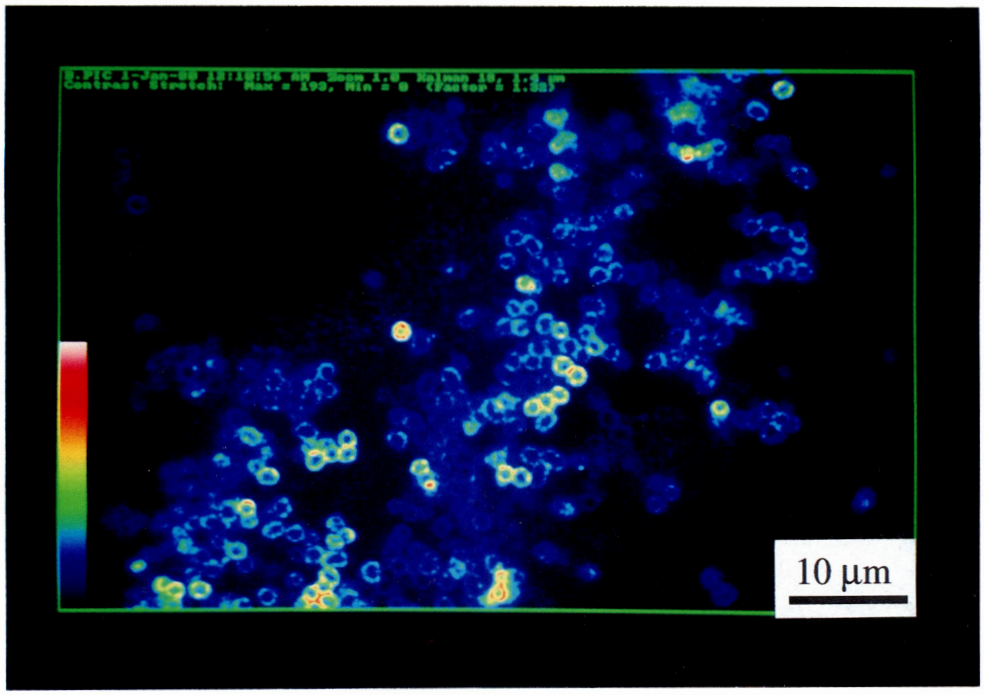

Fig. 3 Optical section images of the poly(MA-NANPOMe-2G) microspheres after they were exposed to the sera of a malaria infected patient in the Philippines. The yellow-red color shows the high intensity of the fluorescence indicating the localization of the NANP-reacted antibodies on the surface of the microspheres. The same pattern of the positive images was shown by the sera from respective malaria patients.

UK). Nine patients' sera at ×16 dilution could show the fluorescence on the surface of the microspheres (Fig. 3), however no fluorescence was shown by the sera from one Japanese imported malaria patient and 10 healthy Japanese donors. The fact that most of the sera from infected patients showed marked and specific fluorescence on the surface of the microspheres supports the assumption that, when the NANP units are in the side chain, they are flexible and adopt a conformation which is accessible for the antibodies, or in other words, recognizable as the antigen to the specific antibodies. At present, however, our data do not provide information needed to describe this particular conformation.

Microspheres have been used increasingly as carriers for biomaterials (Kumakura *et al.*, 1983) and we had previously been preparing microspheres for various applications by radiation induced polymerization (Yoshida *et al.*, 1987; 1990). The advantages of this preparation method are that the synthesis can be carried out without any surfactant or stabilizer, and that it gives monodisperse spheres. The results showed that the polymeric microspheres prepared by the present method functioned as synthetic peptides which specifically bound anti-NANP antibodies developed in malaria patients. Therefore, the most direct application of these results will be a practical assessment of anti-sporozoite antibodies in the humans who contracted malaria. Besides this particular application, a novel method for artificial antigen design has been developed.

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