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# Nanoparticles functionalised with an anti-platelet human antibody for *in vivo* detection of atherosclerotic plaque by magnetic resonance imaging

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## Abstract

Atherosclerosis is an inflammatory disease associated with the formation of atheroma plaques likely to rupture in which platelets are involved both in atherogenesis and atherothrombosis. The rupture is linked to the molecular composition of vulnerable plaques, causing acute cardiovascular events. In this study we propose an original targeted contrast agent for molecular imaging of atherosclerosis. Versatile USPIO (VUSPIO) nanoparticles, enhancing contrast in MR imaging, were functionalised with a recombinant human IgG4 antibody, rIgG4 TEG4, targeting human activated platelets. The maintenance of immunoreactivity of the targeted VUSPIO against platelets was confirmed *in vitro* by flow cytometry, transmission electronic and optical microscopy. In the atherosclerotic ApoE$^{-/-}$ mouse model, high-resolution *ex vivo* MRI demonstrated the selective binding of TEG4-VUSPIO on atheroma plaques. It is noteworthy that the rationale for targeting platelets within atherosclerotic lesions is highlighted by our targeted contrast agent using a human anti-αIIbβ3 antibody as a targeting moiety.
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*Key words:* MRI contrast agents; Atherosclerosis; Nanoparticles; Platelets; Human antibody

## Background

Atherosclerosis is a systemic disorder affecting arterial beds throughout the body, potentially resulting in acute atherothrombotic events such as coronary artery disease (CAD), cerebrovascular disease (CVD), peripheral arterial disease (PAD) or a combination of all (polyvascular or diffuse vascular disease). These cardiovascular diseases cause 19 million deaths per year in the world. They are expected to be the main cause of death globally within the next 10 years owing to their rapidly increasing prevalence in developing countries, due to population aging and other factors, including the increase in unhealthy dietary patterns, physical inactivity, obesity and diabetes mellitus.[1] Thus, the clinical burdent of atherosclerosis is likely to present enormous challenges in the future.

The current opinion is that atherosclerosis is an immune/inflammatory response of the intima to endothelial injury, mainly initiated by the transport of oxidised low-density lipoprotein (Ox-LDL) across the endothelium.[2,3] Several lines of evidence have shown that platelet interactions with modified lipoproteins seem to be quite important in triggering their transfer to the vessel wall.[4] Platelets are by themselves inflammatory cells[5] which can greatly influence monocyte and lymphocyte

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recruitment through interactions with the dysfunctional endothelium in a well-controlled process involving selectins and integrins.[6-8] P-selectin-dependent formation of platelet-leucocyte aggregates (PLAs) further induces the release of a wealth of adhesive and pro-inflammatory substances.[9,10] The process continues in a vicious circle-like fashion and blood cells involved in adaptive immunity may play important roles in the self-perpetuating inflammatory process.[11,12] Monocytes further differentiate into activated macrophages expressing scavenger receptors which bind different forms of OxLDL, leading to lipid-laden foam cells.[13,14] Platelets also act on the stability and vulnerability of lipid-rich plaques, through αIIbβ3-mediated platelet-endothelium firm adhesion, CD40L expression and cytokine secretion[15] which coordinate extracellular matrix proteins lysis by matrix metallo-proteases (MMPs), well-known to degrade and fragilize the fibromuscular cap.[16,17]

Thus, platelets foster an inflammatory environment that influences atherosclerotic plaque development and vulnerability, in addition to their role in acute thrombus formation.[7]

Traditionally, the degree of luminal stenosis has been used as a marker of the stage of atherosclerosis and as an indication for surgical intervention. Coronary angiography is the gold standard technique for lumenography, but unfortunately provides no information about the functional and molecular events leading to plaque rupture.[18] Hence, imaging modalities with more pronostic value are highly desirable.

MRI approaches have successfully characterised carotid arteries, thanks to its high spatial resolution.[19] However, up to now, the tortuosity and size of the coronary arteries added to the respiratory and cardiac motion hinder the *in vivo* imaging of coronary plaque. To overcome these problems and provide information on the molecular and cellular events leading to plaque rupture, we must rely on molecular imaging modalities, capable of reporting on the molecular content of the arterial wall.

In recent years, considerable efforts have been spent in the development of targeted magnetic contrast agents for biomedical imaging in MRI.[20] These must be designed to have no toxicity and selective binding to desired epitopes such as cell surface receptors.[21] With affinities classically in the nanomolar range, antibodies offer binding properties advantages over bio-mimetics and peptides. In order to ensure safety for medical purposes, human antibodies are preferred over murine antibodies (see limitation sections). Moreover, the choice of the targeted biomarker is of fundamental importance because it has to fulfil two criteria: (1) it must sign a pathological state and (2) it should be highly represented.

In light of the above arguments, we believe molecular targeting of platelets is relevant due to their important involvement into every stage of atheroma pathogenesis.

The remaining question concerns their localization and representativity: due to the variety of mecanisms allowing their internalisation – in addition to haemorrhage and thrombi – platelets are certainly retained within the plaque, providing novel means of discriminating atheroma plaques at high risk of rupture.

We thus developed a recombinant human antibody, rIgG4 TEG4, targeting human activated platelets, to be used as a targeting moiety. TEG4 human antibody was obtained through phage-display technology by biopanning on activated platelets.[22] We now produced TEG4 in IgG4 format thanks to the baculovirus-insect cell system, in quantity sufficient to perform biofunctionalisation of nanoparticles. We then designed an original superparamagnetic iron oxide nanoparticle (VUSPIO for Versatile Ultra Small SuperParamagnetic Iron Oxide)[23] (patent FR 2855315 (also published as EP 1627395 and WO 2004107368)) chosen as the contrast agent moiety to covalently couple rIgG4 TEG4 human antibody in order to ensure safety if inoculated in humans.

## Methods

### *Production of TEG4 antibody as a recombinant $IgG_4$ in baculovirus system*

The general principle is to replace a non-essential gene with a DNA sequence encoding a foreign protein of interest. This replacement is promoted by homologous recombination between DNA purified from a replication-defective baculovirus[24,25] and a plasmid called "transfer vector" (pVT). Specific baculovirus cassettes have been designed[26] to express the heavy and light chains of an antibody. These cassettes consist of (i) a strong very late viral promoter (P10 or polyedrin (PH)), (ii) a sequence encoding an immunoglobulin signal peptide, (iii) two unique restriction sites (AflII-NheI for heavy chain expression cassette and BssHII-AvrII for light chain expression cassette) to allow the insertion of the VH or VL sequences of the TEG4 anti-αIIbβ3 antibody[22,27], in frame with the upstream signal peptide sequence, and (iv) a downstream sequence that encodes the human heavy (γ4) or light chain (λ) constant region. These cassettes are flanked by viral sequences that direct the integration of the foreign genes into a specific P10 or PH locus.

Recombinant IgG4 TEG4 was produced from *Sf*9 cells infected with the recombinant baculovirus coexpressing the TEG4 heavy and light chains. Recombinant IgG4 TEG4 was further purified on Protein A column (GE HealthCare Life Science,Velizy-Villacoublay, France). Details of cloning into transfer vectors, generation of recombinant viruses and purification of recombinant antibody are provided in online Supplementary Materials.

### *Synthesis of TEG4-VUSPIO and control-VUSPIO conjugates*

The Versatile UltraSmall SuperParamagnetic Iron Oxide (VUSPIO) platform is based on 7.5 nm-sized magnetic cores (maghemite $\gamma$-$Fe_2O_3$) functionalized by an aminated polysiloxane film grafted on their surface and embedded in a dextran corona. VUSPIO particles differ from USPIO contrast agents by its chemical stability thanks to strong covalent bonds established between magnetic cores and dextran macromolecules. Moreover, their surface is functionalised with long heterobifunctional poly(ethylene oxide) chains serving as cross linkers for derivatisation by fluorochromes and targeting agents.[28,29]

The rIgG4 TEG4 antibody conjugation to VUSPIO contrast agent is achieved by using $SM(PEG)_{24}$ (Thermo Scientific, Courtaboeuf, France) as coupling agent by converting the remaining primary amine terminal groups into maleimide functions. In parallel, a thiolation of TEG4 is performed with

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