**INTRODUCTION**

The noninvasive diagnosis and monitoring of inflammation processes are of particular relevance considering their involvement in a broad spectrum of pathologies, such as infection, rheumatoid arthritis, ischemia, graft rejection, atherosclerosis and formation of tumor metastasis (1–5). In this context, inflammatory cells, such as macrophages, can be tagged *in vivo* with ultrasmall particles of iron oxide (USPIO). Macrophages are indeed able to take up USPIO by phagocytosis, and to subsequently invade tissues through inflammatory processes. This has been achieved in a model of central nervous system (CNS) inflammation (6–8). In this rat model, microglial cells, which are the major intrinsic component of the CNS immune response, internalize USPIO and are detected by *in vivo* MRI (9, 10). However, this type of cellular MRI requires high doses of iron oxide nanoparticles to saturate the macrophages located in lymph nodes, liver, spleen or bone marrow. Labeling of macrophages with USPIO in atherosclerotic plaques has also been achieved (11). Lymphocytes themselves can be magnetically labeled *ex vivo* by fluid phase endocytosis of iron oxide nanoparticles and re-injected in the blood circulation for MRI studies of the *in vivo* trafficking (12). An alternative strategy to image inflammation is to
develop MRI contrast agents specifically targeting an inflammatory cellular marker like the endothelial adhesion molecules. For this molecular imaging purpose, high relaxivities and high specificity are necessary due to the limited number of targets. With Gd-based contrast agents, a huge number of paramagnetic centers are often needed to get to the adequate detection level (13). Owing to their large number of iron atoms, superparamagnetic nanoparticles of iron oxide have strong $T_2$ and $T_2^*$ effects and are therefore detectable at very low concentrations (14, 15). Their usefulness as markers in molecular imaging has been proven in studies on in vitro specific targeting of E-selectin (16).

E-selectin is a transmembrane adhesion glycoprotein expressed on the vascular endothelium during inflammation. Its natural ligand, the sialyl-Lewis$^x$ (sLe$^x$) [Fig. 1(a)] is expressed by leukocytes. The expression of inflammatory adhesion molecules can be transcriptionally upregulated by cytokines, such as interleukin-1 (IL-1) or tumor necrosis factor $\alpha$ (TNF-$\alpha$). In vitro, E-selectin is expressed between 4 to 30 h after stimulation by IL-1 or TNF-$\alpha$, with a peak of expression at 6 h post-stimulation (17). In a previous work, the synthesis of a paramagnetic complex coupled to a mimetic of the sLe$^x$ molecule [Fig. 1(b)] has been described (18). The resulting specific contrast agent has been evaluated in different animal models of inflammation (19–21). Owing to their high payload of magnetic ions and subsequently to their very high ‘particulate relaxivity’, iron oxide nanoparticles are attractive reporters for molecular imaging. In the present study, the synthesis of a new specific superparamagnetic contrast agent, the USPIO-g-sLe$^x$, obtained by coupling the sLe$^x$ mimetic to USPIO is reported as well as the evaluation of its potential as MRI contrast agent, in vitro on human umbilical vein endothelial cells cultures stimulated by TNF-$\alpha$ and in vivo in a mouse model of hepatitis.

**MATERIALS AND METHODS**

**Contrast agent**

The USPIO used was a dextran-coated nanoparticle of iron oxide (crystal size 5–6 nm, overall size 20–40 nm) obtained by the classical Molday method (22). A mimetic of the sLe$^x$ molecule was synthesized as described (18) and branched to the dextran coating of the USPIO. The coupling between the sLe$^x$ mimetic and the nanoparticle coating was realized by a reaction involving epichlorhydrin and the aminated sLe$^x$ mimetic (Fig. 2). Aliquots of 2.5 ml of USPIO (50 mg Fe) were diluted in 10 ml of water and treated with 10 ml NaOH 5M and 5 ml of epichlorhydrin. The mixture was stirred for 24 h at 40 °C and dialyzed. A solution of sLe$^x$ mimetic (10 mg) in 2 ml of water was added to the USPIO–epichlorhydrin suspension (150 $\mu$mol Fe). The mixture was stirred overnight at room temperature and then dialyzed for 48 h (cut-off membrane 12 000–14 000).

**Relaxometry**

The NMRD relaxation profiles were recorded from 10 kHz to 10 MHz on a STELAR field cycling relaxometer (Stelar, srl, Mede, Italy). Additional measurements at 20 and 60 MHz were performed on Bruker Minispec systems (Bruker, Karlsruhe, Germany).

**In vitro studies**

Human umbilical vein endothelial cells (HUVECs) were provided by the Metastasis Research Laboratory of the University of Liège, Belgium (Professor V. Castronovo). HUVECs were cultured in 0.2% gelatin-coated 75 cm$^2$ culture dishes (Greiner, Wemmel, Belgium) using MCDB 131 medium (InvitroGen, Merelbeke, Belgium), supplemented with 20% fetal bovine serum (FBS, InvitroGen, Merelbeke, Belgium), 2 mM of L-glutamine (InvitroGen, Merelbeke, Belgium), 2 mM of heparin (Aventis Pharma S.A., Brussels, Belgium) and 1% antibiotic—antimycotic solution (InvitroGen, Merelbeke, Belgium) (23). Endothelial cell growth supplement (ECGS) (Sigma-Aldrich, Bornem, Belgium) was prepared from the lyophilized powder (5 mg/ml in sterilized phosphate buffer saline, PBS) and added to the medium at a concentration of 50 $\mu$g/ml (10 $\mu$l/ml of medium). To stimulate the expression of E-selectin, cells were treated during 5 h with 10 ng/ml of the pro-inflammatory cytokine TNF-$\alpha$ (17). TNF-$\alpha$ (Alexis Biochemicals,
Lausen, Switzerland) was prepared by dissolving the lyophilized powder in sterile demineralized water (10 μg/ml). This stock solution was then diluted in sterile PBS and brought to a concentration of 0.5 μg/ml. Stimulated and unstimulated cells were scraped 5 h after addition of TNF-α, and were counted using a hemocytometer. After a 5 min centrifugation at 3000 rpm, cells were resuspended at 10^7 cells/ml in complete medium and put in the wells of 96-well microculture plates (Greiner, Wemmel, Belgium) for the incubation with the contrast agents. USPIO or USPIO-g-sLe^x were added to the cells to get a final iron concentration of 4 mM (total volume for the incubation was 200 μl). After 1 h of incubation, microculture plates were centrifuged during 10 min at 1000 rpm to remove unbound contrast agent. Then, cells were washed twice with 200 μl Hank’s balanced salt solution (HBSS). After the second washing, cells were resuspended in 100 μl of 2% gelatin for MR imaging. The concentration of cells for MRI was 2 × 10^7 cells/ml.

**In vivo studies**

All the animal experiments fulfill the requirements of the Ethical Committee of our institution. Hepatitis was induced on NMRI mice (25–35 g) by intravenous (tail vein) injection of 20 mg/kg of the plant lectin concanavalin A (Con A, from Canavalia ensiformis, Jack bean; Sigma-Aldrich, Bornem, Belgium). The animals were anesthetized with 50 mg/kg b.w., i.p. nembutal (Sanofi, Brussels, Belgium), and USPIO-g-sLe^x was injected in the tail vein at a dose of 30 μmol Fe/kg to Con A-treated mice and to healthy mice used as controls. USPIO was also injected at the same dose to healthy and Con A-treated mice as nonspecific control. NMRI mice were provided by the Laboratory of Biology and Embryology of the University of Mons-Hainaut, Belgium (Professor H. Alexandre).

**MR imaging and data analysis**

MR images were obtained on a Bruker AVANCE-200 system, equipped with a vertical 4.7 T magnet. For the *in vitro* studies, a T2-weighted spin-echo sequence was used [TR/TE 3000/15 ms; 24 echo images (from 15 to 360 ms) recollected; FOV 4 cm; matrix 256×256; four acquisitions; acquisition time 28 min 36 s]. Paravision software was used to measure T2 values and signal intensities on cell samples MR images. Relative signal intensity enhancement (SE) was calculated as a percentage of the signal intensity of samples containing non-incubated cells on the 150 ms echo image, using the following formula:

$$(SE) = 100 \times \frac{(SNR)_{incubated \ cells} - (SNR)_{non-incubated \ cells}}{(SNR)_{non-incubated \ cells}}$$

For the *in vivo* experiments, one pre-contrast and several post-contrast axial images of the liver were acquired at different delays (2–120 min) after the injection of the particles [2D gradient echo (GE) sequence; TR/TE 58.8/5.2 ms; FOV 5.5 cm; matrix 256 × 256; flip angle 50°]. Con A-treated mice were imaged 5 h after the induction of hepatitis. Paravision software was used to measure signal intensities (SI) of ROIs chosen in the liver parenchyma on MR images. Signal-to-noise ratio (SNR) was calculated and relative signal enhancement (SE) was obtained with the following formula:

$$(SE) = 100 \times \frac{(SNR)_{post-contrast} - (SNR)_{pre-contrast}}{(SNR)_{pre-contrast}}$$

Color maps were assigned to *in vivo* MR images using the OSIRIS software package.

**RESULTS AND DISCUSSION**

**Relaxivity**

The relaxivity profiles (r1) of USPIO-g-sLe^x and USPIO are represented in Fig. 3. r2 values of USPIO-g-sLe^x and USPIO at 20 MHz and 37 °C are, respectively, 75.5 and
77 s\(^{-1}\) mm\(^{-1}\). At 60 MHz and 37 °C, these values are respectively 73 and 78.6 s\(^{-1}\) mm\(^{-1}\) (Fig. 3). As compared with the parent particles, the branching of the synthetic mimetic of the sLe\(^x\) molecule does not induce a major change of the relaxometric properties above 10 MHz (0.235 T).

**MR imaging in vitro**

HUVECs were chosen because this cell type is able to express adhesion molecules such as selectins or molecules of the superfamily of immunoglobulins after stimulation by IL-1 or TNF-α (17). A T\(_2\)-weighted MR image of PCR tubes containing HUVECs resuspended in 2% gelatin, stimulated or not with TNF-α and after incubation with USPIO-g-sLe\(^x\) or ungrafted USPIO, is shown in Fig. 4. The darkest signal corresponds to stimulated HUVECs after incubation with USPIO-g-sLe\(^x\). Obviously, a larger amount of specifically targeted contrast agent interacts with stimulated HUVECs.

This is quantitatively confirmed by the signal intensities on T\(_2\)-weighted MR images which show that the largest negative enhancement occurs with USPIO-g-sLe\(^x\) and TNF-α stimulated cells (Fig. 5). A slight decrease of signal is, however, observed in control groups. Endothe-
Reticuloendothelial cells, which are part of the reticuloendothelial system, are indeed able to nonspecifically capture USPIO or USPIO-g-sLe\(^x\) by macropinocytosis (24). As expected, the larger \(r_2\) effect is induced by the USPIO-g-sLe\(^x\) on TNF-\(\alpha\)-treated HUVECs, confirming that these stimulated cells are retaining more specific USPIO than unstimulated HUVECs (Fig. 6). The \(r_2\) value of the latter ones incubated with USPIO-g-sLe\(^x\) significantly differs from the values obtained from HUVECs, stimulated or not, and incubated with ungrafted USPIO. In the case of unstimulated HUVECs incubated with USPIO-g-sLe\(^x\), the signal darkening induced by iron oxide also seems to be greater than with USPIO, but not significantly \((p > 0.05)\) (Fig. 5). The grafting of the sLe\(^x\) mimetic, which contains a mannose residue, probably allows for an interaction of the USPIO with some molecules located at the surface of HUVECs. A slight uptake of USPIO-g-sLe\(^x\) as compared to the ungrafted particles can therefore occur.

**MR imaging in vivo**

Figure 7 shows the axial two-dimensional GE MR images resulting from USPIO-g-sLe\(^x\) or USPIO injection to healthy and Con A-treated mice. In our model only hepatic failure is induced and no other organ is injured. Con A binds to endothelial cells of liver sinusoids and induces a T-cell mediated hepatitis (25, 26). E-selectin and other adhesion molecules are then massively expressed on the endothelium of liver vessels (27). A

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**Figure 6.** \(R_2\) values of TNF-\(\alpha\)-stimulated and unstimulated HUVEC samples \((2 \times 10^7 \text{ cells/ml})\), after an incubation with 4 mM USPIO-g-sLe\(^x\) or ungrafted USPIO. Normalization of \(R_2\) values was obtained by removing the \(R_2\) of nonincubated cells. The results are represented as averages \(\pm\) SEM and were statistically processed using Student’s \(t\)-test: **: \(P < 0.01\) as compared to the TNF-\(\alpha\)-stimulated HUVEC samples incubated with 4 mM USPIO-g-sLe\(^x\), xx: \(P < 0.01\) and x: \(P < 0.05\) as compared to the unstimulated HUVEC samples incubated with 4 mM USPIO-g-sLe\(^x\). (“n” is the number of analyzed cell samples).

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**Figure 7.** Axial GE MR images (TR/TE: 58.8/5.2 ms, FOV: 5.5 cm, matrix: 256 \(\times\) 256, flip angle: 50\(^\circ\)) of healthy (A, B) and Con A-treated (C, D) mice 65 min after the injection of 30 \(\mu\)mol Fe/kg of USPIO (B, D) or USPIO-g-sLe\(^x\) (A, C). Color scale used for MR images signal intensities mapping with the Osiris software (E).
signal decrease, induced by the USPIO-g-sLe⁵ or USPIO uptake, can be observed. However, with USPIO-g-sLe⁵, the liver of the healthy mice becomes darker than that of the Con A-treated mice. This suggests that USPIO-g-sLe⁵ are taken up by the Kupffer cells of diseased livers to a lesser extent, probably as a result of their interaction with E-selectin on the vascular endothelium.

Analysis of the MR images (Fig. 8) clearly shows that the relative signal enhancement (negative) measured in the liver of Con A-treated mice and injected with USPIO-g-sLe⁵ is significantly larger than for the other groups after 1 h.

Particles of a size lower than 1000 nm can pass through the fenestrae of the liver sinusoids and are phagocytosed by the Kupffer cells. USPIO are allowed to pass through the liver sinusoidal capillaries but, owing to their small size (20–40 nm), they are less well recognized by the Kupffer cells (28). Murine biodistribution studies, performed by MRI and microscopy, have shown that the cellular uptake of USPIO is not significantly different between healthy and Con A-treated mice, suggesting that the function of Kupffer cells is not altered by ConA.

After injection of USPIO-g-sLe⁵, the relative signal enhancement of the hepatic tissue of healthy mice drops to significantly lower values than Con A-treated mice, even at the earliest time points, suggesting a faster uptake of the USPIO-g-sLe⁵ by the healthy liver (p < 0.05 from 5 to 45 min post-injection). It is known that mannose or N-acetylglucosamine-terminated glycoproteins are quickly taken up by the liver thanks to a specific receptor located on sinusoidal cells (31). The mannose receptor (MR), a C-type surface lectin located at the surface of Kupffer cells and endothelial sinusoidal cells, could be responsible for a rapid capture of USPIO-g-sLe⁵ because of the presence of a mannose residue in the sLe⁵ mimetic molecule (32).

CONCLUSIONS

The mimetic of the sialyl Lewis⁵ has been grafted to USPIO, known as a powerful magnetic reporter. In vitro MR imaging performed on cell samples showed a higher retention of USPIO-g-sLe⁵ by stimulated HUVECs, suggesting that an interaction occurs between the USPIO-g-sLe⁵ and the E-selectins expressed at the surface of endothelial cells activated with TNF-α.

In vivo results show that USPIO-g-sLe⁵ has an interesting paradoxical potential for the in vivo diagnosis of inflammatory diseases. ConA-induced liver inflammation reduces the uptake of USPIO-g-sLe⁵ by Kupffer cells. This unexpected situation is likely to be due to a retention of the specific contrast agent by E-selectin expressed on liver endothelial cells during inflammation. This observation is supported by the signal enhancement of the liver parenchyma. The blood half-life of USPIO is about 24 h in humans and 2 h in murine models. This latter value indicates the fact that USPIO are more easily taken up by liver macrophages in murine models (7). Nevertheless,
the decrease in the liver signal caused by the administration of USPIO-g-SLe\textsuperscript{x} is significantly lower in ConA-treated mice, as compared to all the other cases.

These in vivo and in vitro MRI investigations both showed that the SLe\textsuperscript{x} mimetic allows USPIO to interact with endothelial E-selectin, as demonstrated previously for the SLe\textsuperscript{x} mimetic coupled to Gd-DTPA (19–21). The interest of superparamagnetic iron oxide nanoparticles as compared with the gadolinium-based contrast agents is their excellent superparamagnetic iron oxide nanoparticles as compared to Gd-DTPA (19–21). The interest of superparamagnetic iron oxide nanoparticles as compared with the gadolinium-based contrast agents is their excellent detectability even at relatively low concentrations, which is relevant for molecular MRI applications. Leukocytes and endothelial cell adhesion molecules are becoming targets for the diagnosis of endothelial activation during inflammation, and for therapeutic interventions in diseases where an inflammatory process is implicated (33). The synthetic mimetic could also find applications in the therapeutic targeting of E-selectin, as it was achieved with an SLe\textsuperscript{x} peptide mimetic (34, 35).

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