Study of the Binding Capacity of Heparin Functionalized Magnetic Microparticles for Cardiac Lipoprotein Lipase and their Preliminary Evaluation *Ex Vivo* in Rat Hearts

Veronika Schmitt, Brian Rodrigues, Urs O. Häfeli, Katayoun Saatchi*

Faculty of Pharmaceutical Sciences, University of British Columbia, Vancouver, BC V6T 1Z3 Canada

* E-mail: saatchik@interchange.ubc.ca

Abstract. Magnetic particles were modified with covalently-bound surface heparin (HEPMag). The amount and activity of bound heparin was investigated with the toluidine blue, APTT, Kinetichrome Anti-IIa and western blot assays. The particles bound 40 μg/mg (0.42 U/mg) and exhibited excellent anticoagulant activity. However, the activity was 5x less than that of free heparin. The HEPMag particles were then utilized to measure their lipoprotein lipase (LPL) binding potential and compared to commercially available heparin-bound particles (SiMAG-Heparin). HEPMag particles bound 98% LPL compared to 79% for SiMAG-Heparin particles. In addition, the HEPMag particles retained the LPL much better: Upon incubation with 5 U/mL free heparin, HEPMag particles released only 7% of the bound LPL compared to 67% for the SiMAG-Heparin particles. Being able to capture LPL in the heart might be useful for the investigation of the fat acid metabolism in diabetes. *Ex vivo* retrograde heart perfusion studies in rats with the HEPMag particles showed that LPL could be removed from the coronary lumen. These heparin surface modified parties are thus potential tools to magnetically target the coronary arteries and remove LPL in a selective way.

Keywords: Heparin, magnetic particles, Lipoprotein lipase, Coronary targeting, Diabetes, Heart
PACS: 87.80.Fe

INTRODUCTION

Heparin, a glycosaminoglycan, is best known for its anticoagulant activity, but exerts also other biological effects, both in soluble and in particle-bound form. Kemp *et al.* recently published a review on heparin-based nanoparticle composites and their medicinal applications [1]. In one of their own studies they showed that gold or silver nanoparticles derivatized with diaminopyridinyl-heparin effectively inhibited angiogenesis induced by basic fibroblast growth factor (FGF-2) [2]. Chung *et al.* describe the enhanced angiogenic potential of VEGF administration by a heparin-functionalized nanoparticle-fibrin gel complex [3]. In another investigation Wang *et al.* reported antitumour activity of hybrid heparin-paclitaxel nanoparticles. In mice the heparin-paclitaxel conjugate showed overian tumour growth inhibition similar to paclitaxel, but without any induced weight loss [4, 5]. Chung *et al.* looked into polylactide-co-glycolide (PLGA) nanoparticles surface-functionalized with heparin-conjugated pluronic. With such particles, tumour uptake was 2.2 fold higher than the uptake in the bare particles [6]. Chauvierre *et al.* report that heparin-poly(isobutylcyanoacrylate) copolymers can carry hemoglobin effectively. This work is the first report on surface loading as opposed to the traditional encapsulation [7].

To our knowledge, only two reports describe heparin bound to magnetic particles. Liu *et al.* studied the anticoagulation behavior of PVA-coated magnetic particles with surface-bound heparin. They report that longer spacers between PVA and heparin showed better anticoagulation [8]. Khurshid *et al.* used lysine-coated magnetic particles and bound heparin through ionic bonding. They claim that these particles can be used as potential drug carriers for magnetically targeted cancer treatment [9].

CP1311, *8th International Conference on the Scientific and Clinical Applications of Magnetic Carriers*
edited by U. Häfeli, W. Schütt, and M. Zborowski

We are interested in magnetically targeting heparin to the heart for the basic investigation of the cardiac metabolism in diabetes. In patients with diabetes, cardiovascular disease is the leading cause of death [10-12]. The earliest change that occurs in the diabetic heart involves an alteration in energy metabolism [13]. Normally, glucose utilization provides the heart with approximately 30% of its energy requirements [14], whereas FA contributes approximately 70% of ATP necessary for heart function [15]. Following hypoinsulinemia or insulin resistance in diabetic patients, impaired glucose transport and utilization switches energy production exclusively to oxidation of FA [16]. This dramatic increase in FA influx in the heart has been implicated in a number of metabolic, morphological, and mechanical changes, and more recently, in "lipotoxicity" [17].

Lipoprotein lipase (LPL) is produced in cardiomyocytes and eventually transported into the myocyte for numerous metabolic and structural functions [18]. We have previously shown that subsequent to a decline in glucose utilization by the diabetic heart, myocyte surface bound LPL is rapidly recruited to the luminal surface in search of TG substrates. This increased LPL at the coronary vascular lumen could provide surplus FA to the heart. At present, the pathological consequences of this increase in LPL are unknown [19, 20]. Given the pivotal function of LPL in FA delivery, and FA's contribution in mediating lipotoxicity, limiting FA delivery through this mechanism may be crucial for preventing diabetic heart disease. We have previously shown that subsequent to a decline in glucose utilization by the diabetic heart, myocyte surface bound LPL is rapidly recruited to the luminal surface in search of TG substrates. This increased LPL at the coronary vascular lumen could provide surplus FA to the heart. At present, the pathological consequences of this increase in LPL are unknown [19, 21]. Reduction of LPL levels through targeting the coronary lumen should prevent metabolic switching, and delay lipotoxic initiation of diabetic heart disease. Agents like LPL antibodies [22] and tetrahydrolipstatin [23] can inhibit LPL activity but drawbacks include inhibition of other lipases, inappropriate inhibition of LPL in tissues other than the heart, and unsuitability for long-term *in vivo* studies.

Heparin, with no anti-diabetic actions can displace LPL from its binding sites on cell surfaces [24, 25]. A major disadvantage with this agent, however, is global displacement of LPL. To only replace LPL in the heart and overcome this disadvantage, our goal is to use magnetic nanoparticles with surface-bound heparin for site-specific intracoronary heparin delivery to prevent the enhanced LPL activity in diabetic hearts and potentially modulate lipotoxicity (**Figure 1**).

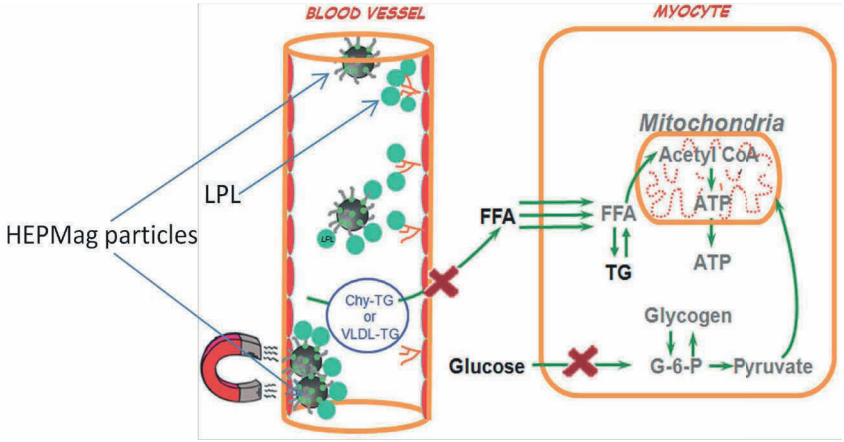

FIGURE 1. Using heparin modified magnetic particles to remove excess LPL from the surface of the coronary lumen might inhibit the generation and cellular uptake of fatty acids.

To successfully replace LPL with magnetic heparin particles, we had to quantitatively know the biological response that such particles display. Different methods were used to measure the activity of surface-bound heparin on home-made (HEPMag) *vs* commercially available (SiMAG-Heparin) particles and compare them to the activity of soluble heparin. The binding and release properties of these particles with bovine LPL, followed by preliminary *ex vivo* studies in the coronary arteries of retrograde perfused hearts of healthy rats are also reported herein.

MATERIALS & METHODS

Chemicals used were obtained from Sigma/Aldrich, Inc. [^{3}H]-triolein was purchased from Amersham, Canada and the heparin sodium injection (Hepalean®; 1000 USP U/mL) from Organon Teknika. Antibodies used for Western blotting were from Santa Cruz. Superparamagnetic polystyrene magnetite particles with terminal NH_2 groups (Estapor®, M2-070/60), the later surface functionalized HEPMag particles, were obtained from Merck Chimie SAS, Fontenay-sous-Bois, France, had an average size of 0.94 μm and contained 65% magnetite. The SiMAG-Heparin particles were from Chemicell, Berlin, Germany and consisted of 1.0 μm diameter non-porous maghemite silica particles surface-functionalized with heparin. The Kinetichrome Anti-IIa Heparin Kit was ordered from Provision Kinetics, Inc. Bovine LPL, used for in vitro incubations, was isolated from bovine milk through separation on a sepharose column [26, 27].

Synthesis of Heparin Coated Magnetic Microparticles

Heparin binding to the magnetite particles: To 200 μl of the Estapor® amine particles was added 200 μl EDC/NHS solution ((N-hydroxy-succinimide, NHS; 3 mg, 26 μmol) and (N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride, EDC; 6 mg, 31 μmol) in 400 μl of 0.1 M 2-morpholinoethanesulfonic acid buffer (MES), 100 μl heparin solution (3.8 mg heparin in 600 μl MES), and 500 μl MES, and the suspension shaken overnight at 25 °C, 1000 rpm. Particles were then washed with H_2O and dried in vacuum.

Determination of Heparin Activity on the Magnetic Microparticles

Toluidine Blue Assay (TB). Toluidine blue (5 mg) was dissolved in 100 ml (0.01 M) HCl solution containing 0.2% NaCl to make a 0.005% stock solution. Heparin sodium salt (4 mg, 202 U/mg) was dissolved in NaCl solution (25 ml, 0.2%) to make a 0.016% standard stock solution. To 5 vials containing 500 μl of 0.005% toluidine blue solution each was added 0, 5, 10, 15, 25, 50, 100, 150 μl of heparin solution (0.01%) to obtain the heparin standard curve. The volume was made up to 1 ml by adding 0.2% NaCl and yielded dark blue to purple solutions. After shaking for 120 s hexane (500 μl) was added, shaken for another 120 s, and centrifuged at 6611 g for 10 min to give a clear solution on the top, a burgundy solid at the interface and a blue solution at the bottom. From the aqueous layer 200 μl was taken and centrifuged again. To 100 μl of the solution 900 μl of ethanol was added, measured in the spectrophotometer at 631 nm and a linear regression standard curve of the heparin concentration constructed. Absorbance readings of the samples were done in triplicates ($R > 0.99$). The heparin content on the particles was measured by adding 1 mg of heparin modified particles in 100 μl MES buffer to a vial containing 500 μl of 0.005% toluidine blue solution. The volume was adjusted to 1.0 ml using NaCl (0.2%) and the suspension shaken for 120 s. Hexane (500 μl) was added, shaken for another 120 s and then 100 μl of the aqueous phase added to 900 μl of ethanol while the particles were held using a magnet. The absorption of the solution was read at 631 nm and the amount of heparin calculated from the standard curve.

APTT Assay. Blood was drawn from a healthy unmedicated donor into an evacuated siliconized glass tube (Becton Dickinson, Franklin Lakes, NJ) containing 3.2% sodium citrate (nine parts blood to one part anticoagulant). Plasma was isolated by centrifugation at 2500 g for 10 min at 4 °C and used immediately. Activated partial thromboplastin time (APTT) was measured with a coagulation analyzer, using mechanical end point determination (ST4, Diagnostica Stago). For the APTT determination, the intrinsic and common coagulation pathways were activated by adding a partial thromboplastin reagent (Dade Behring) and calcium chloride to plasma, and the clotting time was measured. All samples were kept on ice through the experiment.

Heparin particles (1 mg) were dispersed in 200 μl of plasma before use. To 10, 20, 30, 40 and 50 μl of these suspensions in cuvette strips was added 50 μl actin. After 3 minutes incubation at 37 °C, 50 μl of 0.025 M $CaCl_2$ was added and the timer started. Maximum coagulation time read was 120 s, beyond which it was considered as ∞ (non-coagulating). There was a plasma control for every trial with a coagulation time of between 29 to 32 s for all samples. Bare particles (-NH_2 particles) were also tested giving coagulation times of about 30 s, similar to the plasma control. The results were then compared to the behaviour of heparin solutions.

Kinetichrome Anti-IIa Heparin Assay. The chromogenic anti-factor IIa (thrombin) activity in samples containing heparin, low molecular weight heparin and related molecules with thrombin inhibitor activity was measured using a kit and protocol provided by Provision Kinetics. The principle of this assay is the formation of the heparin-antithrombin complex, which is able to inhibit a certain amount of a known excess added amount of thrombin. Remaining thrombin is detected by formation of a chromophore at 405 nm with the thrombin IIa substrate

(substrate). For a calibration curve, heparin dilutions ranging from 0 to 0.025 U/mL were prepared and processed in parallel with samples of SiMAG-Heparin and HEPMag. Absorbance readings of the samples were done in triplicates at 405 nm and two different dilutions each and compared to the heparin standard curve.

Enzyme Activity Measurement and Visualisation of Lipoprotein Lipase

LPL Assay. LPL activity was measured by hydrolysis of [^{3}H]triolein according to a literature procedure [21, 28]. The cleaved oleic acids were extracted and the disintegrations per minute measured using a beta scintillation counter and subsequently converted into units for enzyme activity (nmol/h/mL).

Western Blotting. After SDS-polyacrylamid gel electrophoresis and blotting, the membrane with the separated LPL cleaved from HEPMag and SiMAG-Heparin particles was blocked with 5% non-fat-dry milk and a specific LPL antibody was applied (mouse monoclonal LPL 5D2 antibody, Santa Cruz Biotechnology Inc.). A secondary HRP-conjugated antibody (Santa Cruz Biotechnology Inc.) was then applied, rinsed, and made luminescent by incubation with the enzyme substrate (ECL detection kit, GE Healthcare).

In Vitro and Ex Vivo Experiments with Heparin Coated Magnetic Microparticles

***In Vitro* Binding of Bovine LPL to the Magnetic Particles**. HEPMag and SiMAG-Heparin dilutions were incubated with bovine LPL (0.0118 mg/mL and samples were taken from the supernatant after 15 min (unbound LPL). An excess amount of heparin (100 U/250 μL) was added to competitively release the surface-bound LPL and the supernatant was analyzed for LPL concentrations after 5 and 15 min (released LPL).

***Ex Vivo* Reperfusion**. A modified Langendorff retrograde perfusion procedure was performed as described previously [21, 29] to investigate the *ex vivo* effect of the HEPMag particles. These experiments were performed according to the guidelines of the Canadian Council of Animal Care (CCAC) and the University of British Columbia (UBC). Adult male Wistar rats (280-290 g) were anaesthetized with pentobarbital sodium (60 mg/kg body weight) and euthanised by removing the heart. The hearts were perfused by cannulating the aorta and tying just below the subclavian artery to maintain a recirculation of Krebs buffer containing 10 mM $CaCl_2$ through the coronary blood vessels. In a double-walled, 37°C water-heated beaker, the circulation fluid was gassed with 95% O_2/5%CO_2. The flow rate of the perfusate was adjusted to 8 mL/min. A temporary decrease of the flow rate was necessary during perfusion with particles, to prevent their accumulation in the coronary blood vessels. Hearts were perfused with Krebs buffer containing 2 U/mL heparin (control) or 2 U/mL HEPMag particles, circulating for 10 min. The hearts were then washed with circulating Krebs buffer for 10 min, followed by a perfusion with a 5 U/mL heparin solution to depletively release all bound LPL. Samples were taken after 0, 1, 5, and 10 min.

RESULTS AND DISCUSSION

Both types of heparin-modified magnetic particles showed a relatively smooth surface and did not aggregate, as shown by scanning electron microscopy (Figure 2).

While the non-heparin functionalized Estapor® particles (starting particles) showed no signal in the toluidin blue assay, we measured up to 110 μg of heparin per milligram of derivatized particles. For the following biological tests, we used a batch of HEPMag particles with 40 μg/mg. The heparin concentration of the SiMAG-Heparin particles used for comparison was 9 μg/mg, as measured by the same assay.

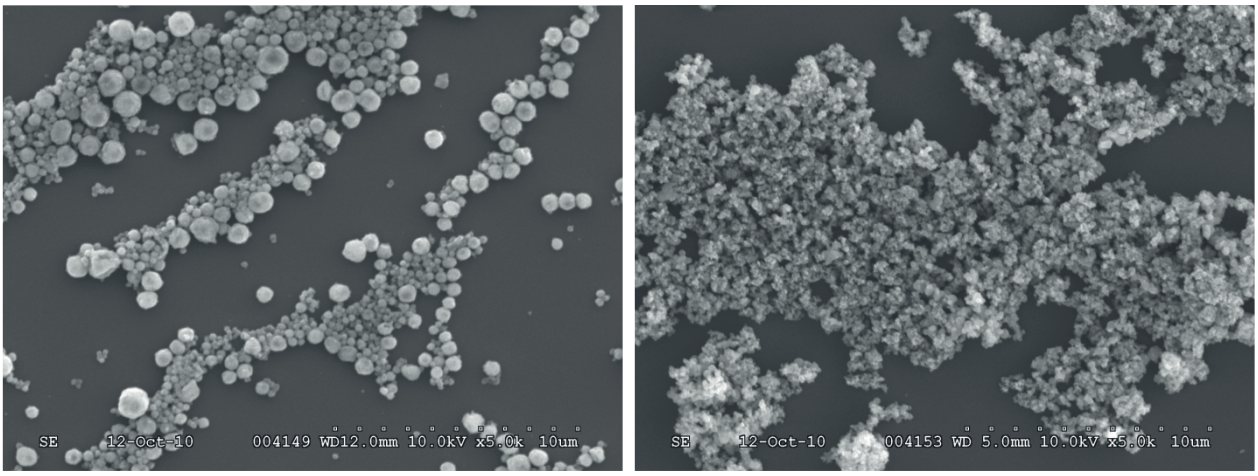

FIGURE 2. SEM of a) HEPMag and b) SiMAG-Heparin particles.

The most commonly used application of heparin is as an anticoagulant, and it is this property that is very often used to confirm the presence and activity of heparin. We used the activated partial thromboplastin time (APTT) to confirm the anticoagulation activity of the particle-bound heparin. The APTT describes the functioning of the intrinsic and common coagulation pathway, and the results are expressed in seconds required for a fibrin clot to form in the plasma after a partial thromboplastin reagent (actin) and calcium chloride is added to the sample. The average coagulation time for normal plasma is about 30 s, shown in the bottom left corner of Figure 3. The unmodified Estapor® particles had coagulation times similar to what was seen for free plasma controls. All heparin-coated particles possessed anticoagulant activity (green symbols). However, the activity of the particle bound heparin (HEPMag) was about 5 times less than that of the free heparin (red symbols) (Figure 3).

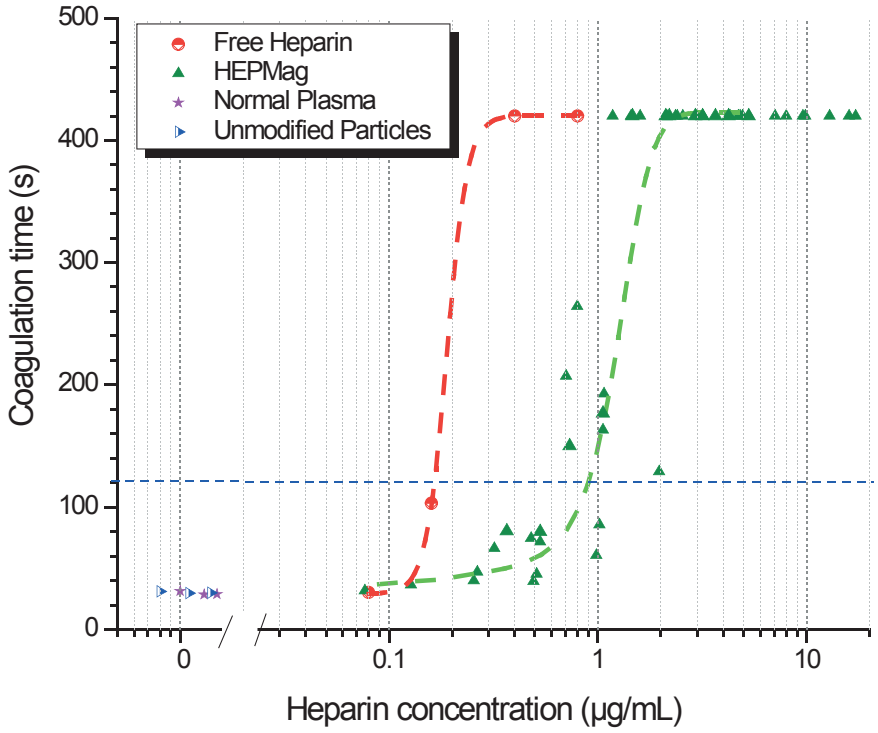

FIGURE 3. Influence of heparin concentration on coagulation time. Normal plasma coagulates in about 30 s, a time which is unchanged by unmodified particles. Free heparin prevents blood coagulation at about 5x lower concentration than the HEPMag particles.

To be able to directly express bound heparin activity per mg of particle, we performed the Kinetichrome Anti-IIa Heparin assay. This assay is commonly used for medicinal purposes. The final concentration of heparin on the surface was 0.00036 ± 0.00008 U/mg for SiMAG-Heparin particles and 0.420 ± 0.030 U/mg for HEPMag particles. The biological heparin activity of the HEPMag particles is thus about 1000 times higher than that of the SiMAG-Heparin particles. Less of the bound heparin on the SiMAG-Heparin particles might thus be available to form the heparin-antithrombin complex than in the HEPMag particles.

As expected, heparin-coated particles were able to bind LPL well. After 15 minutes of incubation *in vitro*, both tested particles were able to reduce the concentration of bovine LPL in the supernatant significantly (Figure 4A). HEPMag reduced the concentration of unbound LPL more than the SiMAG-Heparin particles (98% vs. 79%), but at the same time also retained it much stronger (Figure 4B), as the addition of soluble heparin was not able to release it completely afterwards (7% vs. 67% release). The SiMAG-Heparin particles, however, released almost all of the initially bound LPL in this second step. Based on these observations HEPMag seems to be a good candidate to pick up cardiac lipoprotein lipase directly from the coronary blood vessels as these particles are able to bind the enzyme very strongly on their surface.

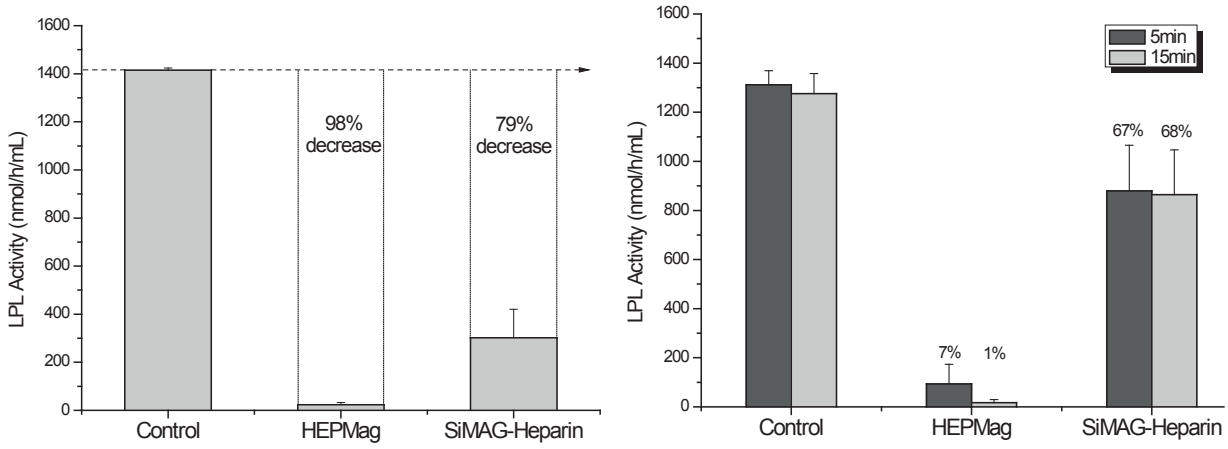

FIGURE 4. A) LPL activity decrease in the supernatant after 15 min of incubation with heparin particles or with soluble heparin (control). B) LPL release from the particles after treatment with excess heparin for 5 or 15 min.

The particle samples with bound LPL before and after incubation with excess heparin to release the bound LPL were also analyzed by Western blotting to determine the LPL concentration. Although not quantitative this experiment clearly confirmed that the HEPMag particles still bound LPL even after 5 - 15 minutes of forced release testing with excess free heparin solution (Figure 5). LPL is still observed in the HEPMag particles of lanes 4 and 6 after incubation for 5 and 15 minutes with excess heparin. LPL is thus very tightly bound. In contrast, SiMAG-Heparin particles have no heparin left on their surface after incubation with excess heparin for 5 minutes (lane 5). All LPL has been removed from the SiMAG-Heparin particles by competitive free heparin after 5 minutes.

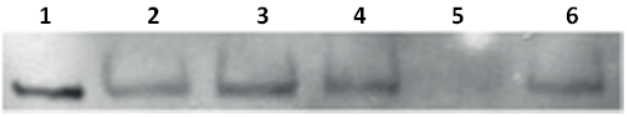

FIGURE 5. From left to right: 1) LPL control; 2) HEPMag after 15 min of LPL incubation; 3) SiMAG-Heparin after 15 min LPL incubation; 4) HEPMag after 5 min release time with heparin incubation; 5) SiMAG-Heparin after 5 min release time with heparin incubation (Note: no heparin left on the particles); 6) HEPMag after 15 min release time with heparin incubation.

As the *in vitro* LPL binding experiments showed a strong binding capacity for the HEPMag particles, we tested the particles directly in coronary blood vessels of retrogradely perfused rats hearts. We calculated to pump an amount of HEPMag particles through the coronary blood vessels that equaled 2 U/mL heparin and compare the release of LPL with a heparin dilution of the same concentration. To reach this heparin concentration with HEPMag particles, the Kinetichrome Anti IIa-Heparin assay results were used (0.42 U heparin per mg particles with a 5x lower activity than free heparin). Using this information, a heparin activity of 2 U/mL was reached by circulating for 10 minutes either 22 mg of HEPMag particles dispersed in 10 mL of Krebs buffer in the heart *vs.* 2U heparin/mL in

10 mL of Krebs buffer (control). The hearts were then washed with circulating Krebs buffer for 10 min and after that perfused with a 5 U/mL heparin solution, to depletively release all remaining LPL from the coronary blood vessels. Figure 6 shows the results of the remaining LPL released with 5 U/mL heparin dilution after pre-perfusion. There is no strong increase of LPL activity between 1 and 10 min, and the curve progression reflects a slow sustained release of a small amount of remaining LPL. A normal LPL release from the coronary blood vessels with 5 U/mL heparin perfusion and no pre-perfusion is shown in blue in Figure 6. It is clearly visible that the curve progression is rapidly increasing between 1 and 2 minutes and after 3 minutes reaches a maximum. Finally both determinations of the remaining LPL release (Figure 6) show similar and comparable curve progressions and activity numbers, resulting in the supposition, that 2 U/mL of HEPMag have a great potential to bind cardiac lipoprotein lipase during circulation in the coronary blood vessels.

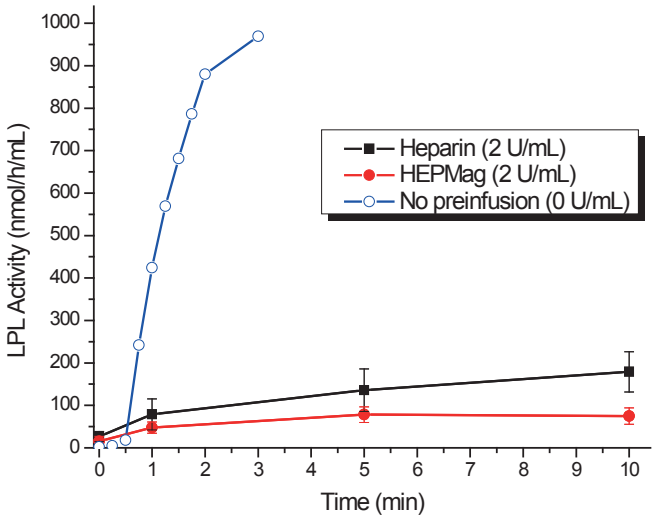

FIGURE 6. LPL activity in supernatant after heart perfusion with 5 U heparin/mL. The hearts *ex vivo* have been either pre-treated with 2U HEPMag/mL (red), 2U Heparin/mL (black) or no pre-perfusion (blue).

These preliminary HEPMag experimental *ex vivo* results in the rat hearts indicate that LPL can be removed from the endothelial cell surfaces and trapped in particles. This would make it possible to investigate cardiac metabolism in diabetic rats locally by magnetically directing HEPMag particles to the heart. For this purpose, a permanent magnet will be placed directly on the chest over the heart, which leads to accumulation of LPL-bound magnetic heparin particles in the capillaries and/or coronary arteries of the heart. LPL could thus be removed from the heart in a defined way, without also releasing LPL from other organs and tissue of the animal (e.g., the muscles). The heparin particles with bound LPL could then be later removed extracorporeally by a process similarly to dialysis. Instead of a dialysis membrane, however, there would be a magnetizable filter which traps the magnetized particles when flowing by and extracts them from the blood.

Further investigations toward the aim of defined extraction of LPL from the heart with magnetic particles are planned. However, there are still some particle-related hurdles to overcome to allow for such experiments. The concentration of particles needed to obtain 2 U/mL heparin was rather high, which seemed to lead to rapid clogging of the coronary arteries in some *ex vivo* experiments. And this despite the fact that the magnetic HEPMag particles did not aggregate under the microscope and, at a size of about 1 µm diameter, were much smaller than red blood cells. It might be necessary to further optimize size, surface coating, surface charge, and infusion concentration.

CONCLUSION AND FUTURE WORK

Magnetite particles were modified with covalently bound heparin on the surface (HEPMag) and characterized by different assays for the heparin quantity and activity versus free heparin in solution. These particles were compared to commercially available particles (SiMAG-Heparin) for their LPL binding properties. HEPMag particles bound LPL strongly without any release and were able to remove the enzyme LPL *ex vivo* from the coronary lumen. Future work will be focused on optimization of perfusion conditions, *in vivo* magnetic targeting of the particles to the heart coronary arteries and further removal of the particles using extracorporeal techniques.

ACKNOWLEDGMENTS

We appreciate the help of Ashraf Abrahani in teaching V.S. the LPL assay, Klaus Freitag for doing the (many) first experiments for this project, and the Canadian Diabetes Association for funding with a grant-in-aid. We are also thankful to Christian Bergemann from Chemicell to provide us with many different types of magnetic SiMAG particles for our research.

REFERENCES

1. M. M. Kemp and R. J. Linhardt, *Wiley Interdiscip Rev Nanomed Nanobiotechnol* **2**, 77 (2010).
2. M. M. Kemp, A. Kumar, S. Mousa, et al., *Biomacromol* **10**, 589 (2009).
3. Y.-I. Chung, S.-K. Kim, Y.-K. Lee, et al., *J Contr Rel* **143**, 282 (2010).
4. X. Wang, J. Li, Y. Wang, et al., *ACS Nano* **3**, 3165 (2009).
5. Y. Wang, D. Xin, K. Liu, et al., *Bioconjugate Chem* **20**, 2214 (2009).
6. Y.-I. Chung, J. C. Kim, Y. H. Kim, et al., *J Contr Rel* **143**, 374 (2010).
7. C. Chauvierre, M. C. Marden, C. Vauthier, et al., *Biomaterials* **25**, 3081 (2004).
8. T.-Y. Liu, L.-Y. Huang, S.-H. Hu, et al., *J Biomed Nanotechnol* **3**, 353 (2007).
9. H. Khurshid, S. H. Kim, M. J. Bonder, et al., *J Appl Phys* **105**, 07B308/1 (2009).
10. A. Lteif Amale, J. Mather Kieren, and M. Clark Charles, *Cardiol Rev* **11**, 262 (2003).
11. J. R. Sowers, M. Epstein, and E. D. Frohlich, *Hypertension* **37**, 1053 (2001).
12. P. W. Wilson, *Endocrinol Metab Clin North Am* **30**, 857 (2001).
13. M. Diamant, H. J. Lamb, Y. Groeneveld, et al., *J Am Coll Cardiol* **42**, 328 (2003).
14. C. Bonadonna Riccardo, *Rev Endocr Metab Disord* **5**, 89 (2004).
15. G. D. Lopaschuk, D. D. Belke, J. Gamble, et al., *Biochim Biophys Acta* **1213**, 263 (1994).
16. B. Rodrigues, M. C. Cam, and J. H. McNeill, *J Mol Cell Cardiol* **27**, 169 (1995).
17. P. Schrauwen and K. C. Hesselink Matthijs, *Diabetes* **53**, 1412 (2004).
18. M. Bergo, G. Wu, T. Ruge, et al., *J Biol Chem* **277**, 11927 (2002).
19. N. Sambandam, M. A. Abrahani, S. Craig, et al., *Am J Physiol Heart Circ Physiol* **278**, H1874 (2000).
20. T. Pulinilkunnil, D. An, S. Ghosh, et al., *Am J Physiol Heart Circ Physiol* **288**, H2802 (2005).
21. B. Rodrigues, M. C. Cam, K. Jian, et al., *Diabetes* **46**, 1346 (1997).
22. I. J. Goldberg, N. A. Le, H. N. Ginsberg, et al., *J Clin Invest* **81**, 561 (1988).
23. A. Lookene, N. Skottova, and G. Olivecrona, *Eur J Biochem* **222**, 395 (1994).
24. D. M. Goldberg, J. G. Parkes, T. Chajek-Shaul, et al., *Adv Enzyme Regul* **31**, 195 (1991).
25. H. Yokoyama, B. Myrup, P. Oturai, et al., *J Diabetes Complications* **9**, 97 (1995).
26. N. Parthasarathy, I. J. Goldberg, P. Sivaram, et al., *J Biol Chem* **269**, 22391 (1994).
27. R. Hayashi, S. Tajima, and A. Yamamoto, *J Biochem* **100**, 319 (1986).
28. B. Rodrigues, M. Spooner, and D. L. Severson, *Am J Physiol* **262**, E216 (1992).
29. N. Sambandam, M. A. Abrahani, E. St Pierre, et al., *Arterioscler Thromb Vasc Biol* **19**, 1526 (1999).