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Cell death via remote heating of microparticles with potential applications in atherosclerosis and thrombosis therapy

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We report a method to cause cell death by remotely heating microparticles by induction heating, this technique could be used to remove vascular deposits and thrombosis. In this preliminary work, we used micrometer size spherical (ferromagnetic) particles and (pure) iron particles to heat remotely macrophages using inductive heating. Iron particles achieved maximum temperatures of 51 ± 0.5 oC after 30 minutes of inductive heating, while spherical particles achieved a maximum temperature of 43.9 ± 0.2 oC (N=6). The therapeutic outcome was determined by monitoring cell re-growth for 2 days following inductive heating treatment. The initial density of cells in the first day prior to induction heating was 105,000 ± 20,820 cells/ml (N=3). 24 hours after induction heating this number was reduced to 6,666 ± 4,410 cells/ml for the spherical particles and 16,666 ± 9,280 cells/ml for the iron particles. The second day the cells grew to 26,667 ± 6,670 cells/ml and 30,000 ± 15,280 cells/ml respectively. Compared to cell cultures with iron and spherical particles that were not subjected to induction heating, we observed a 97% reduction in cell count for the spherical particles and a 91% reduction for the iron particles after the first 24 hours. After 48 hours we observed a 95% reduction in cell growth for both spherical and iron particles. Induction heating of microparticles was highly effective in reducing the macrophage population and preventing their growth.

Cell Death via Remote Heating of Microparticles with Potential Applications in Atherosclerosis and Thrombosis Therapy

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Keywords: remote cell death, microparticles, atherosclerosis treatment methodologies, electromagnetic induction heating, translational research.

Abstract

We report a method to cause cell death by remotely heating microparticles by induction heating, this technique could be used to remove vascular deposits and thrombosis. In this preliminary work, we used micrometer size spherical (ferromagnetic) particles and (pure) iron particles to heat remotely macrophages using inductive heating. Iron particles achieved maximum temperatures of 51 ± 0.5 ºC after 30 minutes of inductive heating, while spherical particles achieved a maximum temperature of 43.9 ± 0.2 ºC (N=6). The therapeutic outcome was determined by monitoring cell re-growth for 2 days following inductive heating treatment. The initial density of cells in the first day prior to induction heating was 105,000 ± 20,820 cells/ml (N=3). 24 hours after induction heating this number was reduced to 6,666 ± 4,410 cells/ml for the spherical particles and 16,666 ± 9,280 cells/ml for the iron particles. The second day the cells grew to 26,667 ± 6,670 cells/ml and 30,000 ± 15,280 cells/ml respectively. Compared to cell cultures with iron and spherical particles that were not subjected to induction heating, we observed a 97% reduction in cell count for the spherical particles and a 91% reduction for the iron particles after the first 24 hours. After 48 hours we observed a 95% reduction in cell growth for both spherical and iron particles. Induction heating of microparticles was highly effective in reducing the macrophage population and preventing their growth.

1. Introduction

Electromagnetic induction heating is the heating of an electrically conducting object (in this case microparticles) by an alternating magnetic field created by a high-frequency alternating current (AC) passing through a coil[1]. Generated Eddy currents on and within the particles lead to Joule heating[1]. Induction heating was first suggested as a mean of therapy[2] in 1957 and it has been proposed for cancer therapy to reduce the size of tumors by specifically targeting tumor cells with nanoparticles, which are then heated to high enough temperatures to cause death of cancerous cells[3-6].

In this manuscript we propose a method for arthrosclerosis and thrombosis treatment that can break up vascular deposits and thrombus using micrometer sized particles heated using induction heating. Atherosclerosis is the cause of several health complications.[7] Prevention and treatment of atherosclerosis continues to fall short. In atherosclerosis chronic inflammation of the arterial wall is caused by the buildup of macrophages, white blood cells, platelets, and other particles that form plaque [8]. Following plaque formation, stenosis and aneurysm occur, eventually plaque may rupture causing acute coronary events [9]. In thrombosis a blood clot is formed inside a blood vessel made from platelets and fibrin [10]. The clot obstructs blood flow, ultimately creating anoxia and tissue death. A clot may also break free becoming an embolus.

Current treatment methodologies for atherosclerosis include life style change, administrating medicines, and surgical interventions[11]. Life style changes and medications largely rely on delaying the progress of plaque buildup rather than removing it. The surgical interventions are limitedly performed for severe atherosclerosis cases[11]. Treatment for thrombosis mainly involved the use of anticoagulant medication. While anticoagulant therapy prevents worsening of thrombosis, it does not remove the thrombus and comes with various risks, including bleeding, recurrence of thrombosis, pulmonary embolism, and post-thrombotic syndromes. A treatment that can reduce the pre-existing vascular buildups and restore the normal circulation without using invasive surgical procedures would be a very useful option.

In the proposed methodology here, two different types of microsized particles, namely spherical magnetic paricles and random shaped iron particles, were used. An induction heating unit was used externally to create an alternating magnetic field strong enough to heat those particles and reduce cell buildup (as shown conceptually in Fig. 1 (a)). Micro- and nano- particles have been extensively studied as carrier devices to deliver drugs or functional materials to target sites and release/actuate the payload in a controlled manner[12-17]. Nanoparticles are widely used for targeted delivery approaches based on their ability to penetrate through the vascular wall to deeper tissue, the ease by which cells uptake them, and minimal physical disturbance they cause cells due to their smaller size. On the other hand, microparticles have been used more frequently for controlled release applications based on biodegradation. It was recently reported that micrometer sized particles can have more efficient binding to the vascular walls than nanometer sized particles[18, 19], making micro-size particles more appealing for our application.

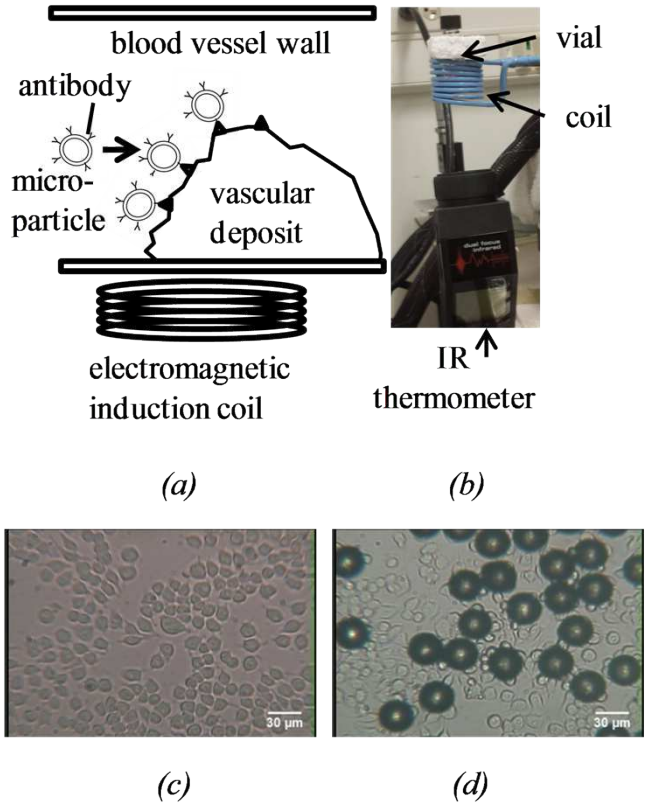

Fig. 1 *(a) is an illustration of micron-sized particles attached on part of vascular deposit site with at least one type of a biological binder, such as an antibody. Inductive heating is used to reduce vascular deposits by heating the targeted vascular deposits at relevant temperatures. (b) The set-up used to heat cells with the micro-particles. (c) Macrophages in vial. (d) Macrophages in vial with spherical particles (diameter 28.0-34.9 µm) attached after 2 hours of incubation (x 200 magnification).*

2. Materials and Methods

2.1. Materials

In this preliminary work we used spherical carboxyl ferromagnetic particles, prepared using chromium dioxide and coated onto uniform polystyrene particles, at a concentration of 0.5% w/v and diameter 28.0-34.9 µm (catalog # CFM-300-5 from Spherotech). We also used pure iron particles < 44 µm (powder Fe-110 from Atlantic Equipment Engineers).

2.2. Cell Culture

A murine macrophage cell line, RAW 264.7, was chosen for the inductive heating study. These macrophages (Fig. 1(c)) were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum and 1% Pen-Strep. For the induction experiments, cells were seeded in 4 mL glass vials (40,000 cells) instead of culture flask.

2.3. Cell Death by Heating

In order to determine the temperature required to achieve necrosis of cells, we placed the vials on a temperature controlled heating plate (Lakeshore temperature controller with 0.1 K accuracy). The temperature was measured with both a thermocouple (Omega) in contact and an infrared thermometer (Infrared Thermometer Dual Focus LS by Micro-Epsilon) remotely. The vials were kept in an incubator overnight to allow surviving cells to grow. The following day macrophage cells were lifted, by trypsinization at room temperature, followed by vigorous flushing. The number of cells in suspension was determined by hemocytometry.

2.4. Cell Death by Induction Heating

Macrophage cells were prepared in the 4 mL vials a day before (40,000 cells / mL input). The following day microparticles particles were added into the cell solution at 0.1 mg/mL final concentration and incubated for a minimum of 2 hours to allow attachment to the cells (Fig. 1(d)). The vials were placed in the center of the induction coil. Induction heating was generated using an Easyheat induction heating system (Ambrell-Ameritherm Co.). A picture of the set-up is shown in Fig. 1 (b). An infrared thermometer was placed under the vial to measure the temperature of the cells and the micro-particles. The frequency used was 350 kHz, the current through the coil was 178.5 A and the power was 1 kW achieving a field intensity of 34 kA/m at the center of the coil. After completing the heating procedure, the cells were kept in an incubator (37 C, 5% CO_2, and nearly saturated moisture)

over night. The following day, the cells were detached from the glass bottom by trypsinization at room temperature and by vigorous flushing with a pipette. The cells were mixed with the equivalent volume of trypan blue solution to discriminate live cells from dead ones and counted with a hemocytometer using an Olympus IMT-2 Inverted Phase Contrast Fluorescence microscope. The process was repeated a day later with the remaining vials.

3. Results

First, we determined the temperature required to achieve necrosis of cells. Vials were placed on a temperature controlled heating plate and the temperature was set to 37 °C, 45 °C and 55 °C for 30 minutes each. The following day there were about 40,000 cells/ml at a temperature of 37 °C, there were 14,000 cells/ml heated to 45 °C and no cells survived (0 cells/ml) at temperatures of 55 °C.

Induction heating results of the two particle types on cell culture in media are shown in Fig. 2. The iron microparticles achieved a maximum temperature of 51 ± 0.5 °C after 30 minutes (N=6), while the spherical particles achieved a maximum temperature of 43.9 ± 0.2 °C (N=6). It should be noted that these values represent the temperature of the entire solution. The heating by induction is instant and spatially confined within the short vicinity of particles. Therefore, the effective temperature at the particle may be much higher than the bulk temperature measured by the IR thermometer.

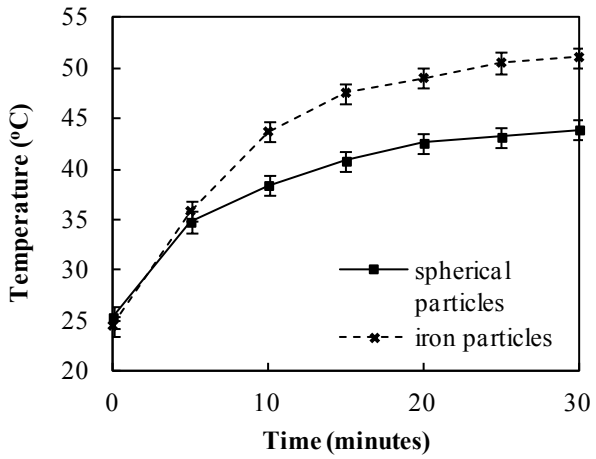

Fig. 2 *Temperature change over time of particles on cells in vials and in the alternating magnetic field with standard error bars (sample number N=6).*

Following induction heating, the cells were incubated. The number of surviving cells were counted with a hemocytometer at 24 hours and 48 hours following induction heating. Two control tests were also performed. First, a batch of cells with particles that were not subjected to heating. This control helped us determine the effects the particles have on the cells such as possible toxicity. The second batch of cells was unheated and did not contain particles.

Fig. 3 (f) summarizes the results. Significant reduction in cell survival was observed in inductively heated cells, compared to the controls, as shown in Figure 3 (a-e). While the initial number of cells the first day prior to heating was 105,000 ± 20,820 cells/ml. 24 hours after induction heating the cell count reduced to 6,666 ± 4,410 cells/ml for the spherical particles and to 16,666 ± 9,280 cells/ml for the iron particles (N=3). 48 hours after induction heating the cell count was 26,667 ± 6,670 cells/ml for the spherical particles and 30,000 ± 15,280 cells/ml for the iron particles respectively (N=3). The cells that were not heated and kept without particles grew to 226,670 ± 23,330 cells/ml after 24 hours and 921,670 ± 78,550 cells/ml after 48 hours. The cells with spherical particles that were not subjected to heating grew to 216,660 ± 42,262 cells/ml after 24 hours and 516,660 ± 76,720 cells/ml after 48 hours. Cells with iron particles that were not heated grew to 188,333 ± 4,409 cells/ml after 24 hours and 648,333 ± 151,116 cells/ml after 48 hours.

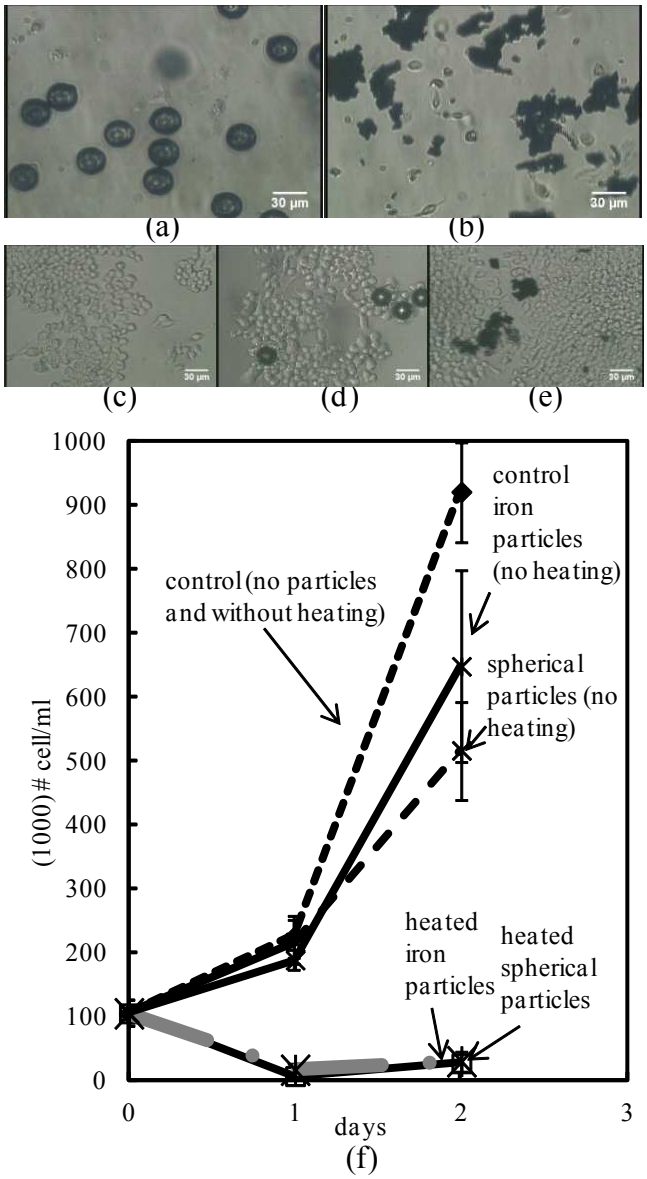

Fig. 3 *(a) Heated spherical particles on cells on day 2 (mag. x 200) (b) Heated iron particles on cells on day 2 (mag. x 200) (c) Control vial on day 2 (mag. x 200). (d) Spherical particles on cells in control vials on day 2 (mag. x 200) (e) Iron particles on cells in control vials on day 2 (mag. x 200). (f) Hemocytometry cell count of cell growth. Results with standard error of mean (N=3).*

4. Discussion

The present study demonstrates that induction heating of two types of microparticles could cause effective damage to macrophage cells, which is a cell line relevant to atherosclerosis. Induction heating has been mostly investigated for cancer therapy. We believe that the same principle should we

studied to treating vascular buildups. Current results provide preliminary information about therapeutic parameters, including the effective particle size, type of particles and possible temperature range.

The highest temperature achieved by spherical magnetic particles was ~ 43 ^{o}C but cell death at this temperature was greater than that achieved by the iron particles, that were heated at higher temperatures (~ 51 ^{o}C). It was observed that spherical particles have a better ability to bind to cells than random shaped iron particles. While most of the spherical magnetic particles attached to cellular surface, iron particles were only partially uptaken by macrophage cells. As mentioned before, the heating of metal particles by induction is confined to the narrow vicinity around the particle. The temperature in the local region could be much higher than bulk temperature we measured. The tighter contact of cells with the spherical microparticles may explain the results. Ultimately, we believe that the local temperature should be high enough to cause cell lysis, not just necrosis. This would ensure reduction of the plaque or thrombus, while avoiding creating an embolus. However, there are a lot of questions that still require answers.

We observed that cells without microparticles exhibited higher growth compared to the controls that included microparticles but were not subjected to heating. This reduction in cell growth may be due to the activation of the phatocytotic activity of the cells rather than the toxicity of the particles, because the exponential growth pattern was still observed.

We chose macrophages as a model cell line that represents the atherosclerotic condition. Macrophages are the main component of arterial plaque along with platelets and white blood cells. Macrophages play a central role in atherosclerosis, including scavenging of modified lipoprotein, generation of foam cells, breaking down / thinning of the endothelial layer (fibrous cap) to turn into unstable lesions, secretion of cytotoxic molecules to promote death of surrounding cells, forming necrotic core, and so on[20-22]. A number of recent studies suggested using macrophage as therapeutic target for artherosclerosis[23-25]. In addition, macrophages are typical phagocytotic cells. In the immune

system macrophages ingest pathogenic microorganisms and have the ability to bind randomly to foreign particles including the micro-particles we introduced. The non-specific phagocytosis process was utilized to simulate cell binding to microparticles. The microparticles used in this study were not modified for targeting.

While this manuscript describes the use of micro-particles to eliminate macrophages by induction heating, we are not suggesting that macrophages are the only component of plaque that needs to be targeted. Plaque has many other components. Specific targeting materials need to be developed. For instance, clot-binding peptide cysteine-arginine-glutamic acid-lysine-alanine (CREKA) can be used to target fibrin, which is present in both plaque and in thrombus[26, 27].

There are additional challenges for instance extensive plaque is usually sheltered with endothelial cells, consequently particles may have to target different types of cells. Smooth muscle cells are also part of established plaques, these muscles provide physical strength and stability, keeping away the hazardous necrotic core from clotting factors in the blood. Therefore healing may cause platelet aggregation. Plaques may disintegrating and either incite an inflammatory response or cause clots to form. These are all very valid problems that need to be addressed. It is for example possible to fabricate microparticles whose half surface is hydrophobic or otherwise not sticky to organic materials such as platelets, while the other half is coated with target antibodies.

Target cells can be specifically aimed to avoid damage to nearby endothelial cells or smooth muscle cells because microsphere heating is very localized. Death of macrophages may increase inflammation and intensify the atherosclerosis process. There are many questions that still remain unanswered such as what is the right number and size of microparticles to avoid creating an embolus etc.

Eventually, surface functionalization of particles will be necessary to deliver the particles to arterial plaque selectively. A biological targeting moiety (antibodies, peptides, aptamers etc.) attached to the particles that will selectively bind to one of the substances that make up vascular deposits would be required when flow is introduced. The methodology for specific targeting to arterial plaque is not well established yet. The microparticles can, for instance, be coated with more than one binder, mimicking monocytes which initiate plaque buildup. In one example, one antibody may aid in rolling adhesion (selectin) and another antibody can be used for stationary adhesion (integrin).

An advantage of induction heating is that the therapeutic efficacy can be activated only where the magnetic field is applied. This localization could minimize the possible side effects of non-specific uptake of particles by other distant organs. Thus the accumulation of particles elsewhere may not be damaging because they will not be heated.

In this introductory work we used commercially available particles. However, microparticles can also be manufactured with microfabrication methods such as surface micromachining to control their size, dimensions, properties and function. The magnetic particles can also be used in combination with (or as part of) a magnetic resonance imaging (MRI) contrast agents for monitoring the vascular deposits with MRI contrast enhancement while the patient is undergoing treatment[28, 29].

In a real life setting microparticle extraction would be necessary. The particles can be extracted from a patient by placing a magnet in proximity to the area of extraction causing the particles to flow out of a blood vessel. Alternatively, the microparticles can be fabricated with biodegradable materials containing magnetic payloads to facilitate clearance from the body.

5. Conclusions

In this manuscript we propose a method for arthrosclerosis and thrombosis treatment that can break up vascular deposits using micrometer sized particles heated using induction heating. An induction heating unit is used externally to create an alternating magnetic field strong enough to remotely heat the particles and reduce cell buildup. Compared to cell cultures with iron and spherical particles that were not subjected to induction heating, we observed a 97% reduction in cell count for the spherical particles and a 91% reduction for the iron particles after the first 24 hours. After 48 hours we observed a 95% reduction in cell growth for both spherical and iron particles. The results indicate that the technique was highly effective in reducing the cell culture population. In this work we attempt to introduce the concept and possibility of using induction heating in conjunction with microparticles. While recognizing that that there are several challenges ahead that can be a subject of future research, we also believe that there are several advantages that this technique may offer and is therefore worth studying further.

Acknowledgments

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Conflict of Interest

The authors declare that they have no conflict of interest.

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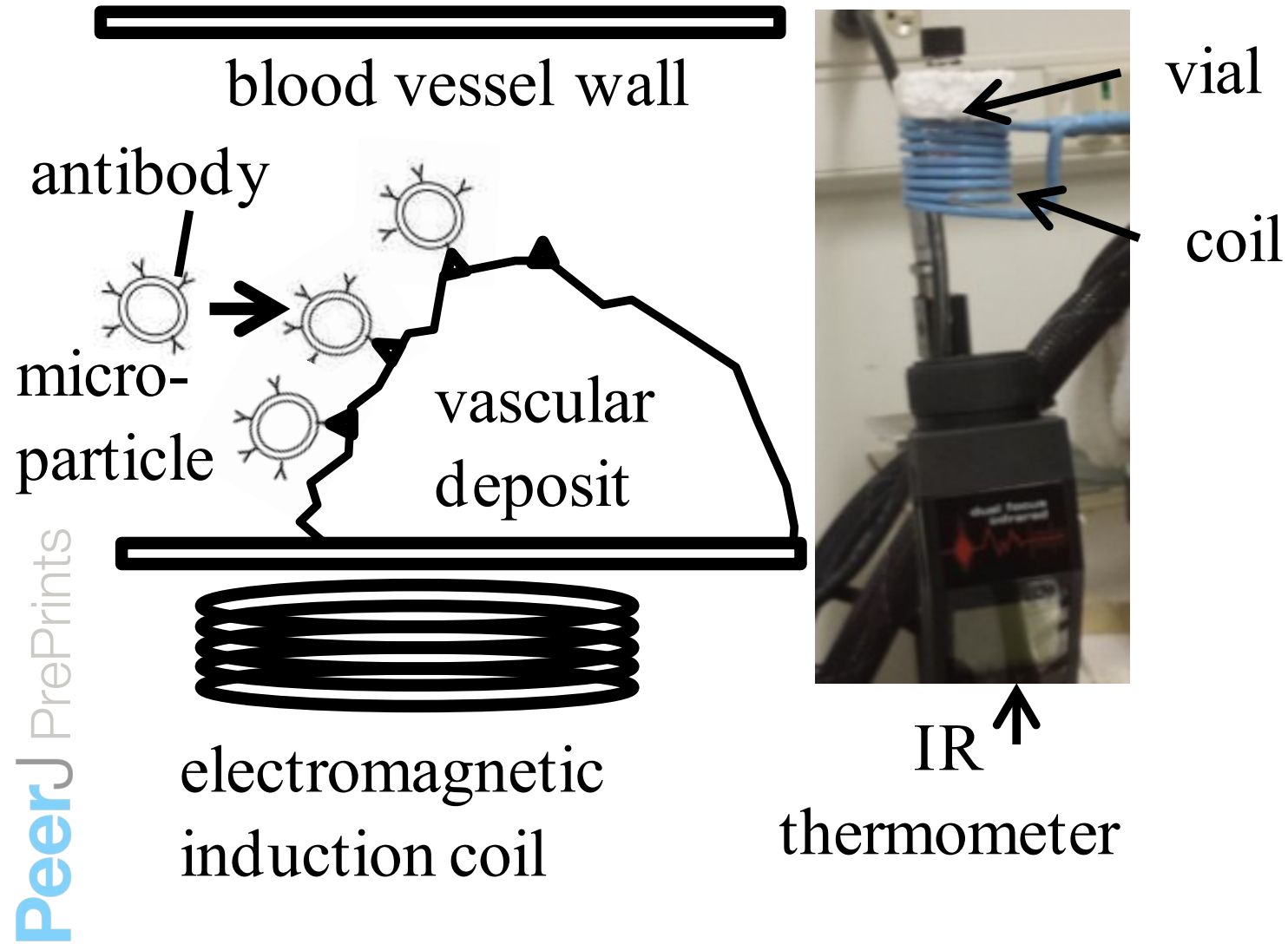
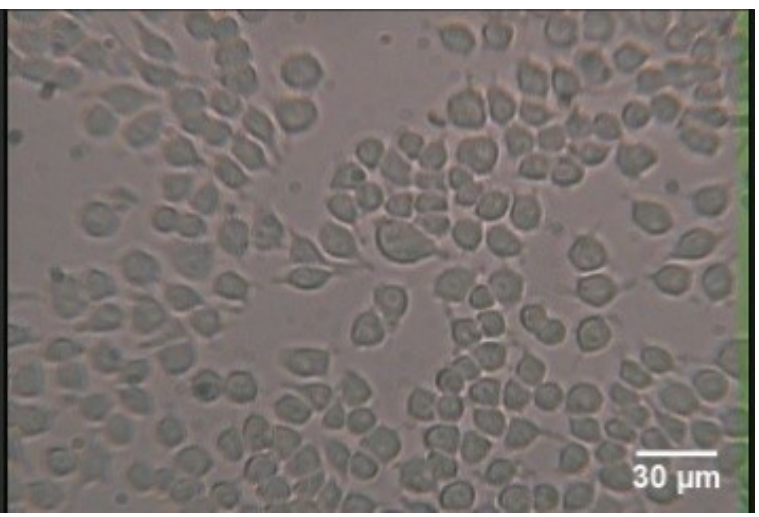
Figure 1(on next page)

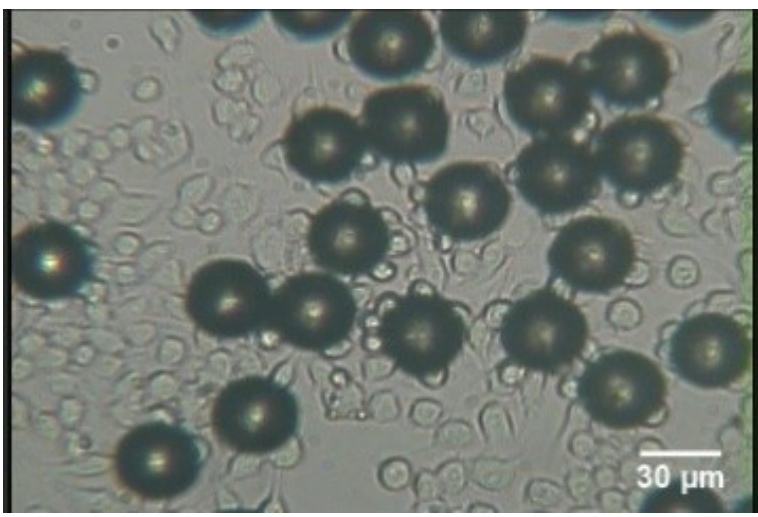
Figure 1

Fig. 1 (a) is an illustration of micron-sized particles attached on part of vascular deposit site with at least one type of a biological binder, such as an antibody. Inductive heating is used to reduce vascular deposits by heating the targeted vascular deposits at relevant temperatures. (b) The set-up used to heat cells with the micro-particles. (c) Macrophages in vial. (d) Macrophages in vial with spherical particles (diameter 28.0-34.9 μm) attached after 2 hours of incubation (x 200 magnification).



(a) (b)





(c) (d)

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Figure 2

Fig. 2 Temperature change over time of particles on cells in vials and in the alternating magnetic field with standard error bars (sample number N=6).

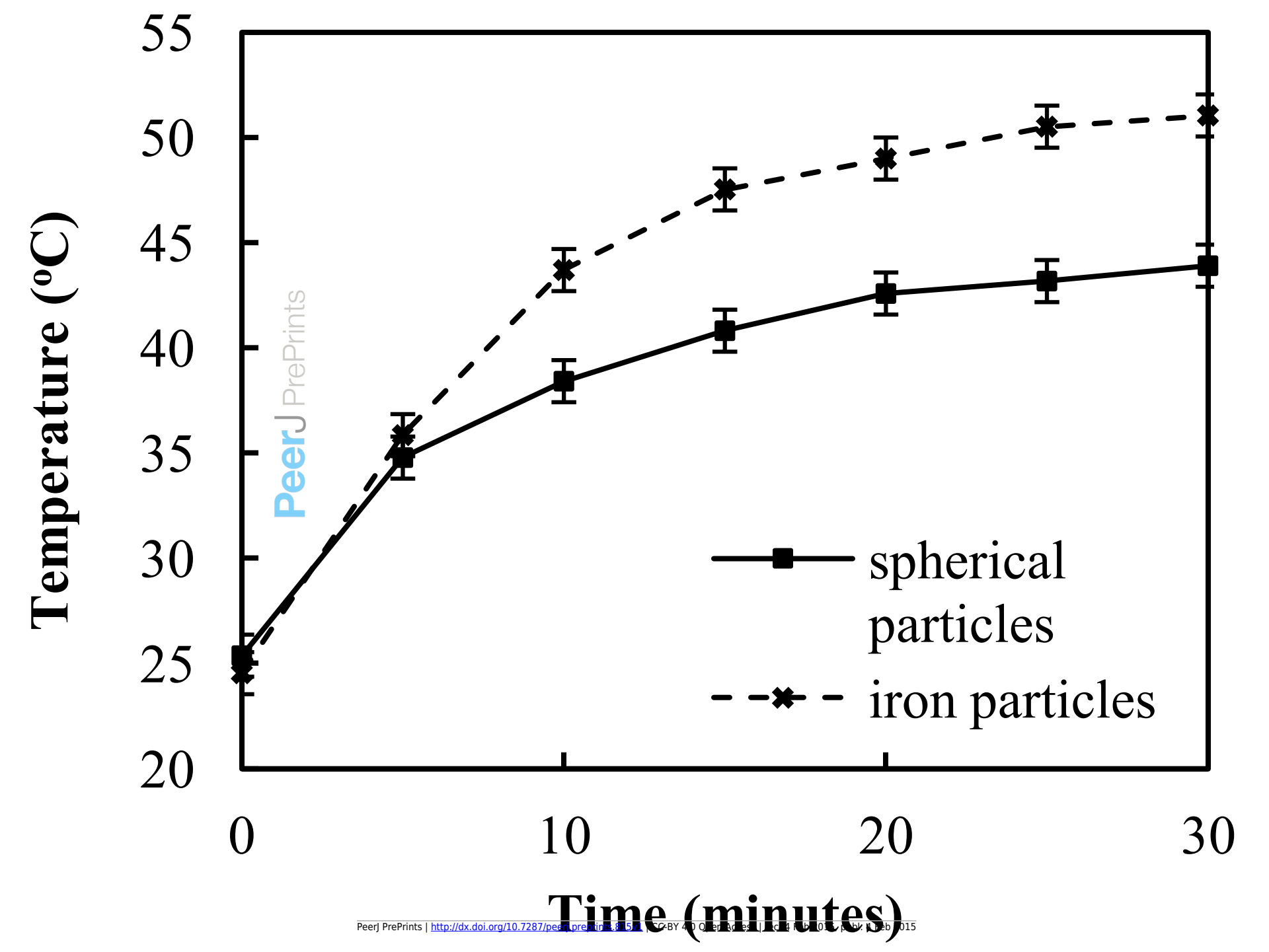

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Figure 3(on next page)

Figure 3

Fig. 3 (a) Heated spherical particles on cells on day 2 (mag. x 200) (b) Heated iron particles on cells on day 2 (mag. x 200) (c) Control vial on day 2 (mag. x 200). (d) Spherical particles on cells in control vials on day 2 (mag. x 200) (e) Iron particles on cells in control vials on day 2 (mag. x 200). (f) Hemocytometry cell count of cell growth. Results with standard error of mean (N=3).