

Biocompatibility and biotolerability assessment of microspheres using a whole blood model

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Abstract

The whole blood model is a powerful method for determining the immediate inflammatory reactions towards foreign objects in general. This review focuses on the use of a lepirudin based whole blood model for evaluating microspheres relevant in cell transplantation applications. This whole blood model can be regarded as a holistic model with readouts from cross-talks between leukocytes, complement, most of the coagulation components and fibrinolysis. A major advantage is the possibility to evaluate a panel of different microspheres under identical conditions, and also the possibility of comparing the reaction patterns between species. This model is a valuable tool for gaining a mechanistic understanding by selected readouts (as complement and coagulation activation products, cytokines, cell-surface receptors, protein adsorption, cell-attachment), and by use of inflammatory blocking agents (inhibitors). The whole blood model is put in the context of today's knowledge about inflammatory systems, discussed according to biocompatibility and biotolerability terms and finally discussed according to its ability to predict the outcome of the transplanted microspheres in an *in vivo* situation.

Introduction

Microencapsulation is an attractive concept to protect transplanted cells against the host immune system. The microspheres should be semipermeable allowing the diffusion of nutrients and oxygen in and cell products out. Although relevant for cell therapy in general, most focus has been on the use of microcapsules for transplantation of pancreatic islets for the treatment of diabetes. The proof of concept has been shown in small [1-4] and larger animal models [5-7].

Clinical phase I studies with three different microencapsulation protocols have been performed in humans [8-10]. Although the clinical trials have concluded that the transplantations are safe and shown function of islets after transplantation, the various protocols have not resulted in a functional cure, e.g. providing enough insulin for the withdrawal of external insulin. One of the major problems after microsphere transplantation is fibrosis reducing the efficacy of the encapsulated islets. The fibrotic overgrowth can be accompanied with some neovascularization, but still with a limited function of the grafted cells.

Due to the complexity of the microencapsulation concept, several factors including the cells [11,12], the transplantation site [13], or the material itself [14-20] are expected to be involved. In order to increase the future clinical success of microencapsulation, it is necessary to reductively study the impact of the various factors. In this review, we focus on a lepirudin based whole blood model as a tool to study the inflammatory potential of the microspheres themselves. The model is unique in the sense that virtually all the inflammatory systems are able to be activated and cross-talk except thrombin dependent activation (discussed later). The model has proved efficient for determining the potential of the microspheres to induce inflammatory mediators as well as revealing the underlying mechanisms. Before turning into the model, we will briefly go into the concept of microsphere technology and discuss the term biocompatibility.

Microspheres

In this review, we are collectively using the term microspheres for microcapsules and microbeads. Microbeads are one-component systems commonly made of alginate cross-linked with divalent cations [21,22]. Microcapsules are composed of two or more polymers forming complexes reducing the permeability. Commonly polycations like poly-L-lysine (PLL), poly-L-ornithine (PLO), poly-D-lysine (PDL), chitosan or poly-methylene-co-guanidine (PMCG) are being applied [22-26]. In addition, some protocols include the solubilizing of the inner core. The properties like stability [27,28], permeability [22,29], cell-compatibility [30-32] and immune-compatibility [14-20] will vary with the microsphere protocol.

Biocompatibility, biotolerability and tolerability

Biocompatibility has been defined as “the quality of being tolerant with life” [33], “the sum of specific and non-specific interactions between blood and biomaterials”³⁴ and “the ability of a material to perform with an appropriate host response in a specific application” [35]. Along the development of the tissue engineering field the latter definition was revised to “the ability of a biomaterial to perform its desired function with respect to a medical therapy, without eliciting any undesirable local or systemic effects in the recipient or beneficiary of that therapy, but generating the most appropriate beneficial cellular or tissue response in that specific situation, and optimizing the clinical relevant performance of that therapy” [36].

In 2011, Ratner published a biocompatibility manifest to challenge the current definitions, since as he states the reactions we call “biocompatible” seems quite the opposite of biocompatible [37]. Since the biocompatibility definitions cause’s confusions, Ratner proposed two definitions “biocompatibility” and “biotolerability”. The new definition of biocompatibility is to describe the biological reactions and the ability to manipulate them. Thus, the definition of biocompatibility would be “the ability of a material to locally trigger and guide non-fibrotic wound healing, reconstruction and tissue integration”. For today’s medical materials we could instead use “biotolerability” to describe a satisfactory outcome for its performance. Thus, definition of biotolerability would be “the ability of a

material to reside in the body for long periods of time with only low degrees of inflammatory reactions”. Along these lines, the microspheres should be defined herein, and the goal should be to have low amounts of fibrotic reactions.

In some cases, like for bone prostheses, it would be advantage to have some degree of inflammation leading to fibrosis which can give a firm adhesion of the material without doing harm. In other cases, like the microspheres, the optimal goal is to obtain a material which is “inert” with respect to the host response. Some would even argue that induction of anti-inflammatory mediators rather than pro-inflammatory could be favorable. If the goal is to obtain an inert material, we disagree in this latter concept, since any response to an agent will imply that the host responds to it, and a number of inflammatory mediators we do not measure or even not know, can be generated as well.

Along the line of tolerability, we would also like to introduce the world complement compatibility [38] which is referring to “the extent the material activates complement”. Compatibility is related to the actual system we are studying. Ideally, the whole body’s response systems should be challenged when studying foreign surfaces. As this evidently is impossible, we have to focus in particular on inflammation and hemostasis, and thus subgroup the definition of biocompatibility to the system(s) we are studying, like leukocyte-, coagulation-, platelet- and complement compatibility. As will be discussed later, complement compatibility could serve as an indicator of microspheres tolerability, and could be an efficient tool for prediction of materials ability to trigger inflammatory reactions, since complement is an important upstream recognition system in first line danger sensing.

Whole blood effector systems

Human whole blood contains important effector proteins and immune cells of the inflammatory machinery. The effector proteins are found in activated products from the complement, coagulation and the fibrinolysis cascades. Complement activation is initiated by target bound antibodies to C1q through the classical pathway (CP), pathogen specific carbohydrates through the lectin pathway (LP) or through binding of spontaneous activated C3 to foreign surfaces as bacteria’s and biomaterials through the alternative pathway (AP). The major steps in complement activation are the formation of enzyme complexes of C3 convertases followed by the formation of the C5 convertases. The CP and LP C3 convertases are identical, composes of C4bC2a, whereas the AP convertase is

composed of C3bBb. When one additionally C3b molecule adds to these convertases, the respective C5 convertases, C4bC2bC3b and C3bBbC3b, are formed. The C5 convertases cleave the C5 to the anaphylatoxin C5a, one of the most potent inflammatory agents leading to a massive inflammatory response [39,40]. C5b leads to formation of C5b-9 which forms the lytic membrane attack complex (MAC), also called the terminal complement complex (TCC).

The coagulation activation is initiated by two different pathways named extrinsic and intrinsic pathway. The extrinsic pathway is initiated by tissue factor (TF). TF is a transmembrane glycoprotein constitutively expressed on extravascular cells (like fibroblasts and pericytes surrounding blood vessels), while inducible expressed upon blood vessel injury [41,42], as well as in monocytes upon stimulation with LPS or bacteria's [43-45]. TF is also exposed on circulating cell-derived microparticles (MPs) [46]. Coagulation factor VIIa complex with TF on the cell membrane and subsequently becomes pro-coagulative upon encountering anionic phospholipids [41]. The VIIa-TF complex initiates the coagulation cascade by activating factor IX and further factor X. The intrinsic pathway, also named contact activating pathway, is initiated by spontaneously binding of factor XII to negatively charged surfaces. The binding converts factor XII to activated factor XIIa, followed subsequently by cleavage of fXI to fXIa and thereafter fIX to fIXa. The contact activating pathway can also be activated by prekallikrein to kallikrein. Both TF and contact activation pathways converge into the common pathway of factor X, which is activated to factor Xa. This again leads to the cleavage of pro-thrombin to thrombin. Thrombin converts subsequently fibrinogen to fibrin leading clot formation. The biological function of the coagulation system is to preserve blood hemostasis, but also to play a role in inflammatory events [47-54]. In addition, platelets play an important role in coagulation as well as in inflammation [55-58].

The hemostatic system also includes the plasminogen-plasmin system with the main function of solubilizing the fibrin-containing thrombi. There are growing evidences for the impact of plasmin in inflammation [59].

The immune cells encodes for a large set of pattern recognition receptors (PRRs) that sense pathogen-associated molecules as well as endogenous molecules released upon stress or tissue damage. Toll-like receptors (TLRs) are a

sub-group of the PRRs with crucial role in the recognition of danger from invading organism or from damaged self. Upon binding of structure specific ligands the TLRs initiates a down-stream signaling ending in induction of cytokines, the key-progenitors of the inflammatory response. Accessory molecules are also needed for the TLRs functioning [60], CD14 being particularly important.

The immune cells are intercommunicating with the effectors from the complement-, coagulation- and fibrinolytic system, thus the systems are highly connected. Examples of the intercommunication are the cross-talk between the TLRs and complement receptors C3aR, C5aR and C5L2 [61,62]. There are also direct communications between the effectors from the plasma cascade systems [47-51], as well as between the platelets and complement [63]. Important aspects of the intercommunication between the various host response systems have recently been reviewed [64-70].

The lepirudin based whole blood model

In whole blood the immune cells, complement, coagulation and fibrinolysis system are able to interact allowing intercommunication. Efficient intercommunication requires functional effector systems, but since a whole blood model needs anticoagulation a complete intercommunication is not possible. The approach has therefore been to affect as few as possible effectors. To inhibit an effector as far downstream as possible would allow intercommunication further up-stream. The most common anticoagulants like ethylenediamineacetic acid (EDTA) and citrate inhibits complement and coagulation by chelating calcium. Calcium is important in most biological processes, including complement and coagulation. By comparing the effect of the four anti-coagulants EDTA, citrate, heparin and hirudin upon activation by lipopolysaccharide in human whole blood, it was demonstrated variable impact by the different anti-coagulants on the chosen readouts (TNF, TF, Platelet factor 4 and lactoferrin) [71]. Hirudin, a highly specific thrombin inhibitor resulted in the overall highest activation of TNF, TF and lactoferrin, thus indicated this anticoagulant allowed the most efficient intercommunication between leukocytes and the effector proteins. In contrary, the secretion of platelet factor 4 was lowered by hirudin compared to heparin [71], thus demonstrating an inhibitory effect of lepirudin on platelets activation.

A whole blood model based on the hirudin analogue lepirudin was published in 2002 [72]. As for hirudin, lepirudin is specifically interacting with thrombin and inhibits its further action. Comparison of the effect of lepirudin (hirudin) and heparin on complement, showed that heparin spontaneous activated complement in low doses and inactivated serum complement in high doses, whereas lepirudin had no effect on complement. For studies of complement and leukocyte interactions, lepirudin is therefore the anticoagulant to be selected since all the complement proteins are still able to function. Besides the critical aspects of the anticoagulant, to be further discussed in the next paragraphs, the treatments of samples to be investigated are also important. The effector proteins from blood, including components of the complement and coagulation cascades can be easily activated by sampling and experimental equipment's. Low activating plastic vials of polypropylene is therefore used, and to avoid further activation after experiment completion, EDTA is added to inactivate complement and coagulation. The plasma is stored at -80°C straight after sampling, and repeated freezing and thawing is avoided to maintain complement and cytokine activities. Readouts in this model are in principle any inflammatory parameter in plasma, as well as cell surface markers analyzed by flow cytometry immediately after incubation. A schematic presentation of the practical accomplishment of the whole blood method is shown in Fig. 1. An overview of methods to measure complement has recently been described [73].

The lepirudin based whole blood model has its limitations by blocking thrombin. Thrombin is not only important in coagulation, but is also the main platelet activator [63,74]. In addition, platelets contains a panel of molecules associated with inflammation [58], and the thrombin receptor seems to be the most important for release of the inflammatory components stored in platelets granules [75]. Platelet functions are therefore influenced by lepirudin. For evaluation of alginate microspheres intended for transplantation at other sites than blood, a reduced involvement from platelets is however preferable. In addition, initial coagulation activation up-stream thrombin, can still be measured, including thrombin cleavage products [76]. Tissue factor, the main inducer of the extrinsic pathway, is still inducible on monocytes using lepirudin as anti-coagulant [45] while not by soluble heparin [77]. Thrombin might have some impacts on leukocyte performance, as monocytes chemotactic responses [78] and IL-8 secretion from PBMCs [79]. In contrary, monocytes are not producing the pro-inflammatory cytokines IL-6 and TNF upon thrombin stimulation and seem to lack the thrombin receptor [80].

Alternative whole blood models with possibilities of preserving the whole panel of effector components in blood are highly warranted. One such alternative is heparin-coated surface used for incubation of non-anticoagulated whole blood. Several models of heparin coats have been published of which the covalently end-point attached method has proved to be superior [81]. Ionic bindings have been tried, but difficulties in getting a complete coating and leakage of heparin from the surface has hampered this technique (personal observation). The lack of global access to the covalently attached heparin for experimental use has limited the progress in this field. Still important papers using non-anticoagulated blood has been published with heparin-coated surfaces decades ago [82-86]. The lepirudin based whole blood model seems currently to be the easiest accessible and most physiological alternative for studies involving complement-cytokine axis, as well as coagulation events up-stream thrombin.

Experiences with the lepirudin based whole blood model

The lepirudin based whole blood model has been highly effective for study the various mechanisms involved in bacterial blood infection [45,72,87-91]. Since the model in principle keep all inflammatory systems able to interact, selective inhibition of the various effector mediators, has led to an increased understanding of particularly the impact of complement on the various effectors in inflammation triggered reactions. In addition, these studies have contributed to reveal the interplay between complement effectors and the PRRs, through studies focusing on the key TLR accessory molecule CD14, in combination with C3 or C5 inhibition [68].

The whole blood model has also been used to gain increased understanding of the inflammatory reactions observed during cardiopulmonary bypass (CBP). Like the concept with microencapsulated cells for transplantation, the increase in inflammation seen in cardiopulmonary bypass might have a multitude of causes like surgical trauma, ischemia and reperfusion injury, as well as the membrane oxygenator. The whole blood studies of polyvinyl chloride bypass tubes did show that the complement activation and various inflammatory cytokines was activated by the tube themselves [92-94] thus demonstrating that complement incompatibility could at least partly be causing the inflammation found during CBP. Lately the lepirudin based whole blood model has also been used to investigate the

inflammatory potentials of the materials intended to use in glucose sensors [95,96]. Also in these studies it was demonstrated that the C5a was the mediator for cytokines as well as for TF expression on monocytes [96].

The combination of human genetic complement deficiency of C5 and the lepirudin based whole blood model has also given the opportunity to directly assess the role of complement [89,97]. These studies have confirmed this experimental model to be useful for application of specific inhibitors.

Microspheres studies in the lepirudin based whole blood model

The lepirudin based whole blood model has been used to study the inflammatory responses of various alginate containing microspheres [32,98]. These studies and presently unpublished data showed a clear connection between the microspheres ability to activate inflammatory mediators and their formulation. Polycations in form of PLL or PMCG induced complement activation, whereas alginate did not [32]. Since the activation product Bb was increased whereas C4d was not, the activation was dependent on the alternative pathway. Despite the lack of C4d, this does not exclude that a modest initial activation by the classical or lectin pathway could have preceded the alternative activation [99-102]. Elevated levels of the anaphylatoxins C3a and C5a [32] indicate that inflammation would be triggered, since the C5a is a broad stimulator of inflammatory events [40].

The complement activating microspheres induced pro-inflammatory cytokines (TNF, IL- β , IL-6), chemokines (IL-8, MIP-1 α , MCP-1) and the growth factor VEGF [98]. In contrary, alginate microbeads did not increase these mediators, consistent with the lack of complement activation. By inhibiting complement at the C3 level by using compstatin [32] the direct connection between cytokine stimulation and complement was evaluated. The inhibition of C3 completely abolished the secretion of the pro-inflammatory mediators (TNF, IL-1 β , IL-6, IL-8, MCP-1 and VEGF) showing that these mediators were totally dependent on complement [98]. Blocking the TLRs accessory molecule CD14 did not result in any diminished response, again indicating that complement alone was responsible for the microsphere induced cytokine secretion. Also in consistence with complement activation the anti-

inflammatory mediators IL-1ra and IL-10 were induced. This indicates that a truly inert material is not initiating the anti-inflammatory mediators, since they also are produced as a response to the material.

In addition to measure inflammatory mediators in the fluid phase, surface staining might provide important information. C3c is the major C3 protein fragment, present in the native protein and its activation products C3b and iC3b, the mechanisms thoroughly described in [103]. C3 is the most central protein in the complement cascade, and the major component of C3 and C5 convertase. Antibodies to C3c exist and are a valuable tool for surface studies of complement activation. C3c accumulates on PLL containing microcapsule surfaces while not on surfaces of alginate microbeads [32,98], indicating that the C3 convertase is established on the polycation containing microsphere surface in contrast to the alginate. An example of C3c deposited on PLL containing alginate microcapsules is shown in Fig. 2. The spotted pattern of C3c in Fig. 2 is probably a result of the amplification of the alternative pathway escalating the establishment of C3 and C5 at their initial activating points.

Cell-adhesion can efficient be studied on microspheres surfaces by the whole blood model. Cell-attachment was consistent with complement activation [31], thus cells attached to polycation containing microcapsules whereas not to alginate microbeads. Potential target points for cell-adhesion in the lepirudin based whole blood model is most likely the activated C3b and its inactivated counterpart iC3b which functions as ligands for the leukocyte receptors CR1 (CD35) and CR3 (CD11b/CD18).

A special case of microspheres are the PMCG microcapsules consisting of poly(methylene-co-guanidine) in complex with cellulose sulfate [26]. A fast complement (TCC) and earlier detectable C3c was found for PMCG compared to PLL containing microcapsules [32]. By time, a lower complement response and no accumulation in C3c on surface was however detected. In consistence with this, the overall cytokine responses were lower for the PMCG microcapsules as compared to the PLL microcapsules. The anaphylatoxins C3a and C5a were hardly detected, which can be explained by surface adsorption. The anaphylatoxins are small positively charged fragments (C3a PI 9.7 and C5a PI 8.6) and might be attracted to the negatively charged cellulose sulfate in outer coating. The anaphylatoxins

can still upon adsorption to a surface be biologically active and promote cell-adhesion to the biomaterial surface. These findings illustrate the necessity of investigating the biomaterial surface in addition to measuring the activators in the fluid phase [104]. Another, yet unexplored aspect with the PMCG microcapsules, are their impact upon coagulation activation. One of its components, cellulose sulfate has been shown to interfere with plasma kininogen [105]. Since the deposition of C3c was low on the PMCG microcapsules surface, we cannot yet exclude the possibility of complement activation through cross-communication by coagulation activation products [50,51]. Ongoing studies will clarify whether the PMCG microcapsules or some of the other types of microspheres have a potential to activate coagulation pathways.

The human lepirudin (hirudin) whole blood model can apart from mechanistic studies also be used to screen various microspheres under identical condition, thus eliminating the uncertainty of individual variations. A broader panel of alginate microspheres analyzed by us indicates that the various factors as alginate source, type of polycation, as well as microspheres protocol will impact their inflammatory potentials (to be published). We would also recommend that round-robin tests of the various microspheres used by different groups to be compared to validate their inflammatory potentials under identical conditions. Also, the model can be used to design new variants of microspheres with improved properties. Comparative studies of the responding pattern in various animal models are also possible, as to predict if the particular animal model will give comparable responses to the human immune effectors. Such studies are currently ongoing with collaborators.

Relevance to the *in vivo* situation

In animal models after transplanting microspheres containing PLL in the peritoneal cavity they are often subjected to more overgrowth than alginate microbeads [14-20], thus the *in vivo* findings are in overall consistent with the complement and cytokine responses in the lepirudin based whole blood model. Microsphere's inducing complement activation and cytokine release in the whole blood model will most likely also be able to initiate the same inflammatory machinery after transplantation.

The complement activation product C5a, which was elevated by the PLL containing microcapsules in our studies, is shown to be involved in fibroblast activation, extracellular matrix deposition and macrophage influx in a renal fibrosis model [106]. This indicates that complement is involved in fibroblast overgrowth and deposition of extracellular matrix.

Complement and coagulation proteins are present at other sites than in blood, such as in the peritoneal fluid ^{107,108}. Upon surgery complement activation products are increased [109], showing that complement easily is activated. Adipocytes found in fat stores produce complement and could be a source of complement components in the peritoneal cavity [110,111]. The adipose tissue also contains endothelial cells, fibroblasts, leukocytes and macrophages [112], as well as expressing TLRs [113], thus the cell effectors that can respond to complement activation are present.

The chemokine IL-8 has been shown to be a strongly complement dependent mediator in both bacterial [91] and biomaterial studies [94]. IL-8 is a strong chemoattractant for neutrophils, abundantly present in circulation and an early responder in inflammation. The T-lymphocytes, basophiles and endothelial cells also have receptors for IL-8 with influence on chemotaxis [114] and angiogenesis [115]. IL-8 has also been shown to have a bimodal function by recruiting neutrophils in early stages and inducing fibrotic and tissue granulation in later stages [116]. It is therefore likely that IL-8 might be an important effector in fibrotic reactions to biomaterials.

At present, our data in the whole blood model is not consistent with *in vivo* findings in every case. The alginate microbeads can in some animal models like the C57/B16 mice be subjected to fibroblast overgrowth, although in lower amounts as the PLL microcapsules (unpublished observations). Also, the intermediate complement and cytokine response of the PMCG microcapsules in the whole blood model was not reflected in a baboon model evaluating overgrowth [117]. These discrepancies could be caused by species differences with respect to the response, or by mechanisms not yet explored in relation to microspheres in the whole blood model.

Concluding remarks

The lepirudin based whole blood model is well suited to explore microspheres inflammatory potential and to predict their tolerability. The whole blood model can be regarded as a short-time screening assay of microspheres and their separate components. The model can be used to gain a mechanistic understanding of the initial factors triggering inflammation, as well as to design new microcapsules with a lower inflammatory potential. The model represents an efficient screening assay for biomaterials tolerability, which in the end concerns the patient safety and the function of the transplanted cells.

Conflicts of interest

There are no conflicts of interest connected to this mini-review. Financial contribution related to this mini-review has been from the Central Norway Regional Health Authority, grant from the European Commission EP-7 BetaCellTherapy, grant from the Slovak Research and Development Agency under the contract No. APVV-0486-10, an EFDS New Horizons grant and by National Institutes of Health grants A1068730 and The Chicago Diabetes Project (<http://www.chicagodiabetesproject.org/>).

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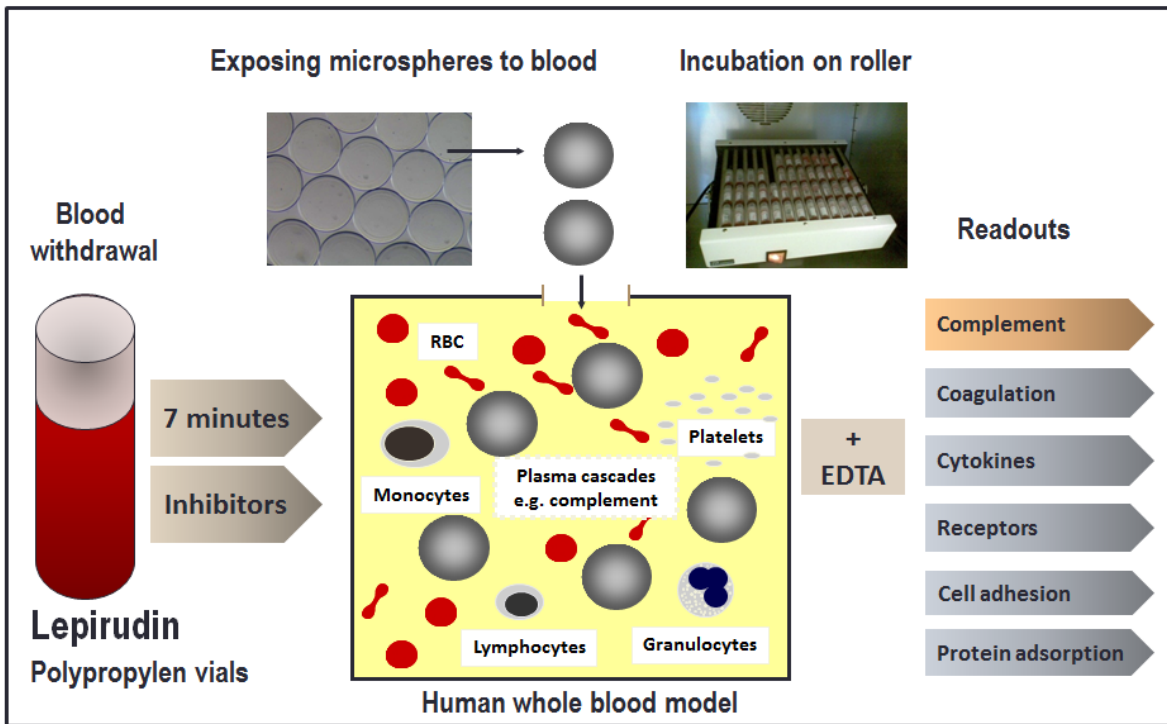


Fig. 1.

Schematic overview of the lepirudin based whole blood model. Blood is withdrawn in polypropylene vials containing lepirudin. Thereafter the blood is transferred to polypropylene vials containing the microspheres. In the cases of inhibition, blood is pre-incubated with or without inhibitors for 7 minutes before addition to microspheres. The vials are incubated on a roller to maintain continuous and careful mixing of blood components. After 15-360 minutes, depending on the readouts, complement and coagulation is inactivated by addition of EDTA. EDTA is not added in the case of cell-adhesion or integrin expression studies. Plasma is frozen immediately upon harvesting.

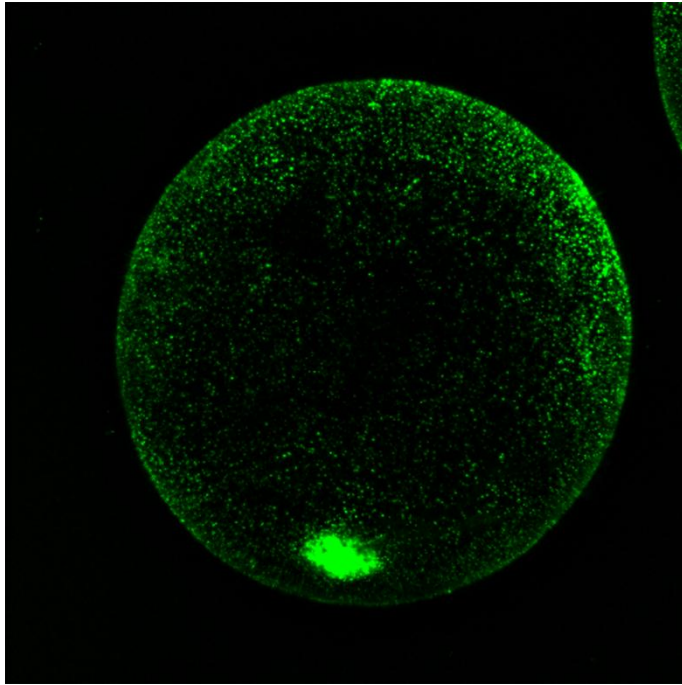


Fig. 2.

Example of the outer surface of a poly-L-lysine containing microcapsules with C3c deposition. The picture is a 3D projection taken by confocal microscopy and FITC-anti C3c staining. C3 is the most central protein in the complement cascade, and the major component of C3 and C5 convertases. C3c is the major fragment of the C3 protein. The spotted patterns indicate that the binding of C3 leads to the binding and conversion of more C3 to C3bBb (C3 convertase), amplifying the alternative pathway, and C3bBbC3b (C5 convertase) triggering the terminal pathway.