

The zebrafish embryo as a model to quantify early inflammatory cell responses to biomaterials

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Received 14 January 2017; revised 4 May 2017; accepted 9 May 2017

Published online 6 June 2017 in Wiley Online Library (wileyonlinelibrary.com). DOI: 10.1002/jbm.a.36110

Abstract: To rapidly assess early inflammatory cell responses provoked by biomaterials in the full complexity of the living organism, we developed a zebrafish embryo model which allows real time analysis of these responses to biomaterial microspheres. Fluorescently labeled microspheres with different properties were injected into embryos of selected transgenic zebrafish lines expressing distinct fluorescent proteins in their neutrophils and macrophages. Recruitment of leukocytes and their interactions with microspheres were monitored using fluorescence microscopy. We developed a novel method using ImageJ and the plugin ObjectJ project file “Zebrafish-Immunotest” for rapid and semi-automated fluorescence quantification of the cellular responses. In the embryo model we observed an ordered inflammatory cell response to polystyrene and poly (ϵ -caprolactone) microspheres, similar to that described for mammalian animal

models. The responses were characterized by an early infiltration of neutrophils followed by macrophages, and subsequent differentially timed migration of these cells away from the microspheres. The size of microspheres (10 and 15 μ m) did not influence the cellular responses. Poly (ϵ -caprolactone) microspheres provoked a stronger infiltration of neutrophils and macrophages than polystyrene microspheres did. Our study shows the potential usefulness of zebrafish embryos for *in vivo* evaluation of biomaterial-associated inflammatory cell responses. © 2017 Wiley Periodicals, Inc. *J Biomed Mater Res Part A*: 105A: 2522–2532, 2017.

Key Words: zebrafish embryo, early inflammatory cell responses, biomaterial microspheres, material properties, *in vivo* imaging

How to cite this article: Zhang XL, Stockhammer OW, de Boer L, Vischer NOE, Spaik HP, Grijpma DW, Zaat SAJ. 2017. The zebrafish embryo as a model to quantify early inflammatory cell responses to biomaterials. *J Biomed Mater Res Part A* 2017;105A:2522–2532.

INTRODUCTION

Evaluation of biocompatibility is an essential step in development of biomaterials. Routinely, cytotoxicity is first assessed *in vitro* in assays with isolated cells. Subsequently *in vivo* tests are performed in relevant mammalian models to assess functionality and to analyze tissue responses in histology.^{1–3} Infiltration of inflammatory cells, predominantly neutrophils and macrophages, in the surrounding tissue characterizes the early phases of the inflammatory response and reflects the extent of tissue compatibility of implanted biomaterials.^{1,3,4} Although histological evaluation provides substantial evidence of inflammatory

cell types and numbers in the vicinity of biomaterials, it is usually time consuming and costly. Because of the need to restrict the use of experimental animals, histological evaluation often is only performed at a limited number of time points, and is performed at a late stage of biomaterial development. In case the biomaterials eventually fail at this stage, this causes financial losses and serious delays in time to market. Moreover, since the animals need to be sacrificed for evaluation, the assessment of the progression of inflammatory cell responses over time in single animals is not possible by traditional histology.^{1,2}

Additional Supporting Information may be found in the online version of this article.

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Recent advances in mouse experimental models have allowed noninvasive characterization of over time cellular responses to biomedical implants *in situ* in single animals, utilizing fluorescent probes specific for neutrophils and macrophages.³ However, these models generally do not allow studies at the microscopic level on cell interactions with materials *in situ*, and they are not intended for high throughput screening. Therefore, we sought to develop a complementary *in vivo* model which would allow the desired real time analysis of early inflammatory cell responses to biomaterials at an early stage of the development of these materials.

Zebrafish (*Danio rerio*) embryos are a powerful *in vivo* system that has been widely used for intravital visualization and analysis of host responses including innate immune response to bacteria and drugs, and to study biomaterial nanotoxicity.^{5–8} A range of transgenic zebrafish lines expressing distinct fluorescent proteins in inflammatory (and other) cell types has been developed.^{9–13} The optical transparency of zebrafish embryos allows real time visualization and analysis of cellular responses using fluorescence imaging techniques. The power of this *in vivo* system has been shown in studies on neutrophil and macrophage responses to tailfin injury of embryos,¹³ migration path analysis of neutrophils,¹⁴ and real time recording of phagocytosis of bacteria by macrophages and/or neutrophils.¹⁵

From a biological point of view, the zebrafish embryos are highly suitable for studying innate immune responses. The innate immune system of zebrafish and mammals is highly similar, comprising neutrophils and macrophages as the major cells types, equally capable of phagocytosing cell debris, apoptotic cells, and microbes as their mammalian counterparts.^{9,16–18} Moreover, orthologs of important mammalian chemokines and cytokines as well as similar signaling pathways of cell migration/recruitment are present in zebrafish.^{14,17,19}

From an automation point of view, the high fertility of zebrafish provides the possibility to obtain the large numbers of embryos required for high throughput systems featuring automated robotic microinjection and imaging techniques.^{20,21} Last but not least, the easy and relatively cheap maintenance of zebrafish reduces the costs to at least 100-fold lower levels than for mice.²²

Based on these advantages, we aimed to utilize zebrafish embryos to develop an *in vivo* model for rapid quantitative analysis of early inflammatory cell responses to microspheres as model biomaterials, using fluorescence imaging techniques. To the best of our knowledge, this is the first study to report the cellular responses to implanted biomaterial microspheres and assess the effect of their material properties on the provoked responses in zebrafish embryos.

MATERIALS AND METHODS

Fluorescent microspheres

Monodispersed poly (ϵ -caprolactone) (PCL) microspheres loaded with the fluorescent dye Coumarin 102 were prepared by the oil in water (o/w) single emulsion membrane emulsification technique,²³ in a fume hood under aseptic conditions. A 10% (w/v) PCL (Molecular weight = 65,000 g/mol, Sigma

Aldrich) solution in dichloromethane (DCM, Merck) containing 0.1% (wt) Coumarin 102 (Dye content = 99%, excitation wavelength λ_{ex} : 387 nm, emission wavelength λ_{em} : 470 nm, Sigma Aldrich) was filtered through a 0.45 μ m sterile polytetrafluoroethylene (PTFE) filter for purifying the polymer solution, and subsequently pushed through a microsieve™ membrane with uniform 11 μ m pores (Nanomi B.V. The Netherlands) to form polymer droplets with an identical size. The formed droplets were dispersed in 4% (w/v) poly vinyl alcohol (PVA) solution in ultrapure water by vigorous stirring for approximately 3 hours at room temperature, allowing evaporation of the DCM. The hardened microspheres were washed repeatedly with ultrapure water containing 0.05% Tween 20 and collected by centrifugation, redispersed in fresh ultrapure water and stored at 4°C. Fluorosphere® polystyrene (PS) microspheres (10 and 15 μ m in diameter, blue fluorescent— λ_{ex} : 365 nm, λ_{em} : 415 nm, Molecular Probes, Life technologies) were purchased. Before use, microspheres were collected by centrifugation, washed repeatedly with sterile phosphate buffered saline (PBS) and re-dispersed in fresh PBS.

Characterization of microspheres

The diameter of PCL microspheres was determined using a Coulter Multisizer (Multisizer 3 Coulter Counter, Beckman Coulter Electronics). The aperture diameter of the capillary used for the size measurements was 100 μ m. The fluorescence intensity of the PCL and PS microspheres was examined *in vitro* using a fluorescence stereo microscope (LM80, Leica). Bright field and fluorescence images of the microspheres were recorded. The particle size of the microspheres was characterized using a scanning electron microscope (SEM, Leica). All SEM specimens were mounted on metal stubs and sputter-coated with gold (Polaron 5000 sputtering system). All images were captured under a tension voltage level of 2 kV and a working distance of approximately 6 mm.

Zebrafish husbandry and collection of embryos

Adult zebrafish were handled in compliance with the local animal welfare regulations approved by the local animal welfare committee (DEC) and were maintained according to standard protocols.²⁴ To allow real time visualization of macrophage or neutrophil responses separately, zebrafish transgenic (Tg) lines with green fluorescent macrophages (mpeg1:Gal4-VP16xUAS:Kaede)¹⁰ or neutrophils (mpo:eGFP)¹² were used. To be able to study the combined responses of macrophages and neutrophils Tg lines with green fluorescent macrophages (mpeg1:Kaede)¹⁰ and with red fluorescent neutrophils (lysc:Dsred2)¹¹ were pair-wise crossed to breed a Tg line with both distinct fluorescent cell populations (mpeg1:Kaede x lysc:DsRed2). Alternatively, Tg lines with red fluorescent macrophages (fms:Gal4i186Xunmi149:mCherry)¹³ and with green fluorescent neutrophils (mpo:eGFP)¹² were pair-wise crossed. After harvesting, zebrafish embryos were maintained in E3 medium²⁴ at 28°C. The E3 medium was refreshed every day. Dead or malformed embryos were removed daily.

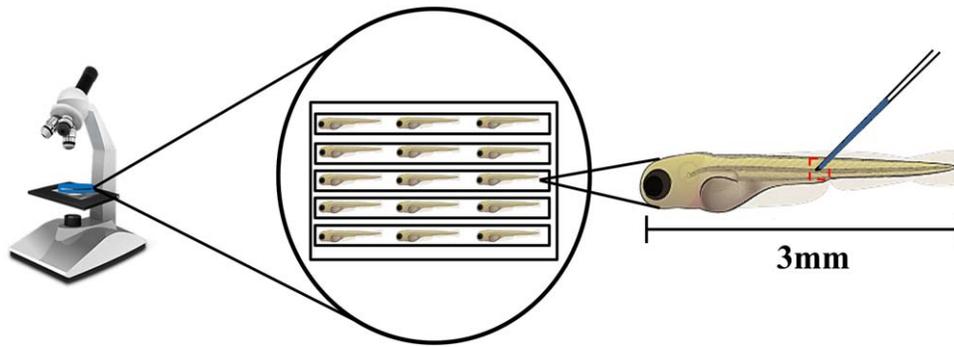


FIGURE 1. Schematic representation of the injection procedure of microspheres into the tail tissue of zebrafish embryos, aligned in a grooved agarose plate under a light microscope. Icons (open source): <http://www.softicons.com>; <http://zebrafishart.blogspot.nl/>.

Injection of microspheres into the tail tissue of zebrafish embryos

The injection procedure is schematically depicted in Figure 1. Zebrafish embryos of the chosen Tg lines were selected and dechorionized at 2 or 3 days post fertilization (dpf). The embryos were aligned in U-shaped grooves in an agarose plate submerged in E3 medium, and anesthetized with 0.03% (w/v) tricaine (buffered 3 aminobenzoic acid ethyl ester, Sigma-Aldrich) added to the E3 medium. The microspheres in 100 μ l of microsphere suspension were collected by centrifugation, washed repeatedly with PBS and dispersed in 100 μ l of 4% (w/v) Polyvinyl Pyrrolidone (PVP, Applichem) solution in PBS. This suspension was loaded into a glass microcapillary (Harvard apparatus, pulled by a flaming micropipette puller (P-97, Sutter Instrument)) connected to a FemtoJet microinjector (Eppendorf). The outer diameter of the tip opening of the microcapillary was manually adjusted to 15 or 20 μ m, by breaking under a light microscope guided by a microruler (LM20, Leica), to suit the injection of 10 μ m and 15 μ m microspheres, respectively. Two to 3 nl of 4% PVP solution containing microspheres was injected into the tail tissue of zebrafish embryos using the FemtoJet microinjector under a light microscope. The majority of embryos received 1 to 3 injected microspheres, some received 4 to 5, and only a few received 6 to 8 microspheres by one injection. According to the product information, the concentration of PS₁₀ microspheres and of PS₁₅ microspheres in suspension was 3.6×10^3 and 1.0×10^3 microspheres/ μ l, respectively. The concentration of PCL₁₅ microspheres was similar, but was not measured. Under the same conditions, 2 to 3 nl of 4% PVP solution without microspheres (designated as PVP solution) was injected. A group of nontreated embryos (NT) that were only anesthetized was used as the control for PVP injection. The numbers of embryos in each group with injections were initially between 23 to 30 and decreased to 23 to 20, due to exclusion of embryos that died during the experiments if any. The numbers of embryos in NT groups were 5 and no embryo died during the experiments.

For confocal microscopy, a single 10 μ m PS microsphere was loaded on the tip of a glass microcapillary with a diameter slightly smaller than that of the microsphere, and pushed into the tail tissue of zebrafish embryos at 2dpf,

using a Narashige IM-11 injector. This alternative procedure was used to have maximal control on localized positioning of the microsphere for confocal imaging.

Image recording using fluorescence microscopy

Zebrafish embryos were anesthetized with 0.03% tricaine and mounted in 2% (w/v) methylene cellulose (Sigma Aldrich) for imaging. Sequential images were recorded under bright field and with FITC, mCherry and UV filters at a magnification of 160 times. The fluorescent Kaede protein expressed by macrophages in the zebrafish Tg line (mpeg1:Kaede) has been reported to undergo photoconversion from green to red fluorescence under illumination with ultraviolet light (350–400 nm).²⁵ However, this photoconversion depends on energy level and period of illumination, and was not observed under the settings used in our study (Supporting Information Fig. S1). A Z stack of 20 μ m in depth with a step size of 10 μ m was applied, allowing to take 3 consecutive images with the focus plane for the middle image set at the microspheres or tissue injury (in case of PVP injection or in the controls). Each individual zebrafish embryo was imaged once every day from 5 hours post injection (hpi) until 4 days post injection (dpi) using a fluorescence microscope (Leica, LM80). Dead embryos were excluded for further image recording since they were dead. A series of time laps images recording the infiltration of both neutrophils and macrophages in response to 15 μ m PCL microspheres between 1 to 2 hpi were taken at a magnification of 100 times and converted into a movie (Supporting Information Video S1) using ImageJ.

Image recording using confocal microscopy

Zebrafish embryos were anesthetized with 0.03% tricaine, mounted to the bottom of a MatTek Glass Bottom Culture Dish (P35G-1–20-C) by covering them with 1.5% (w/v) low melting point (LMP) agarose solution in demi water, allowed to solidify at room temperature. A series of time laps images recording the infiltration of neutrophils in response to a 10 μ m PS microsphere between 1 to 2 hpi were taken with a confocal microscope (SP5, Leica), and the images were converted into a movie (Supporting Information Video S2) using ImageJ.

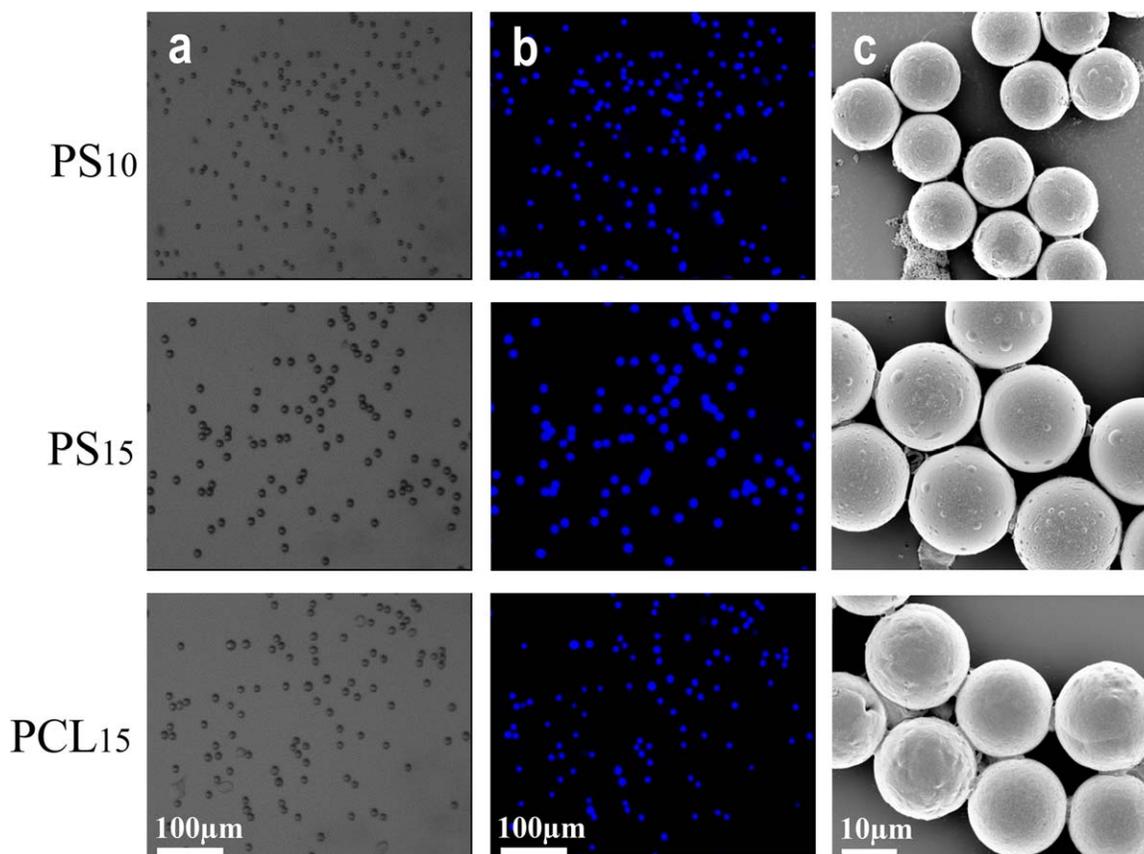


FIGURE 2. Characterization of microspheres by microscopy. (a) Bright field microscopy; (b) Fluorescence microscopy; (c) Scanning electron microscopy.

Fluorescence quantification of inflammatory cell infiltration using ImageJ and ObjectJ

To quantitatively analyze fluorescence intensities corresponding to cell infiltration, we developed an ObjectJ project file called “Zebrafish-Immunotest”, which runs under ImageJ. ObjectJ and “Zebrafish-Immunotest” are documented at and downloadable from the respective following links: <<https://sil.s.fnwi.uva.nl/bcb/objectj>> and <<https://sil.s.fnwi.uva.nl/bcb/objectj/examples/zebrafish/MD/zebrafish-immunotest.html>>. The procedure for fluorescence quantification using Zebrafish-Immunotest is as follows and illustrated in Supporting Information Figure S2. The images recorded under bright field and with FITC, mCherry and UV filters are arranged as four-channel hyperstacks. In the project file, the chosen images are registered as “linked” [Supporting Information Fig. S2(a)]. The injection site of individual embryos is manually marked based on the extent of tissue injury observed in the bright field image [Supporting Information Fig. S2(b)]. Within a radius of 50 μm from the injection point, Zebrafish-Immunotest then detects the highest peak in both the green and red channel. Peak detection is preceded by temporary Gaussian smoothing with $\sigma = 10$ pixels. Either peak position in the two channels is chosen as the center of a standardized area with a diameter of 100 μm for recording the integrated fluorescence, which quantifies the macrophage and neutrophil infiltration in the green and

red channel, respectively [the yellow circle in Supporting Information Fig. S2(c,d)]. The diameter of 100 μm was selected since it typically covered the tissue with most of the local macrophage and neutrophil infiltration in response to injected microspheres, and did not include circulating cells in the blood stream of embryos at the late time points (indicated in Figs. 4 and 6 in the Results section). Data are visualized in individual result columns for Kaede (green) and DsRed (red) with direct access of statistics and histograms or export to spreadsheet programs [Supporting Information Fig. S2(e)]. The parameters of Zebrafish-Immunotest/ObjectJ can be freely changed by users to fit the setup of their studies (detail can be found in the link provided above).

Statistical analysis

The fluorescence quantification (arbitrary units) of neutrophil and macrophage infiltration of every embryo in each group at each time point was plotted individually. According to the Shapiro-Wilk normality test and Kolmogorov-Smirnov test, the values of integrated fluorescence did not (always) follow a Gaussian distribution. Therefore, the nonparametric Kruskal-Wallis test was performed to assess whether differences existed between groups within an experiment (p values < 0.05). Subsequently, differences between pairs of groups were analyzed with the Mann-Whitney test. All analyses were performed using Prism graphpad 5.0. The results were considered significantly

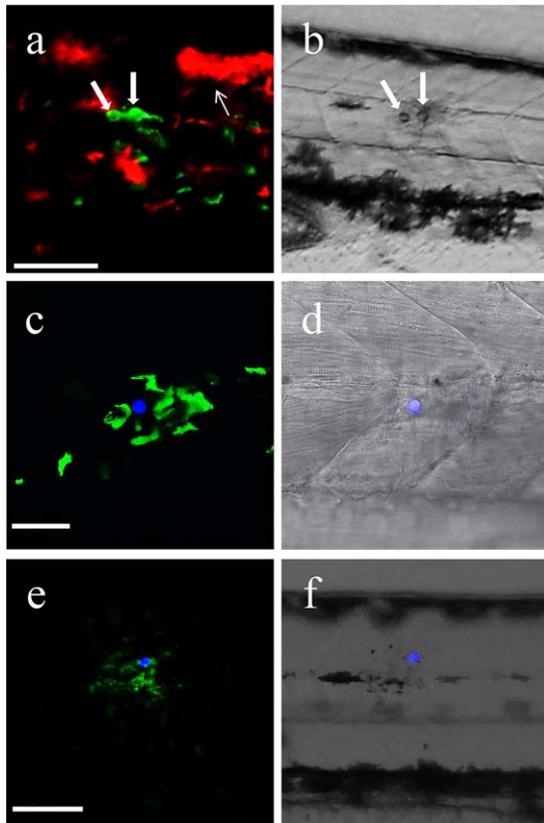


FIGURE 3. Inflammatory cell infiltration in response to injection of microspheres into the tail tissue of zebrafish embryos. a) Fluorescence image recording of the accumulation of eGFP-labeled neutrophils (green) and mCherry-labeled macrophages (red) in the proximity of PCL₁₅ microspheres, between 1 to 2 hours after injection into a 2 days old embryo. Scale bar = 100 μ m; b) Bright field image corresponding to a). The thick arrows in a) and b) indicate locations of the microspheres in the tail tissue after injection. The thin arrow indicates mCherry-expressing pigment cells. These images are part of Supporting Information Video S1; c) Confocal fluorescence image recording of the accumulation of eGFP-labeled neutrophils (green) in the proximity of a PS₁₀ microsphere (blue), between 1 to 2 hours after injection into a 2 days old embryo. Scale bar = 50 μ m; d) The blue fluorescent image of c) combined with the corresponding bright field image. The fluorescent images are part of Supporting Information Video S2; e) Fluorescence image recording of the accumulation of Kaede-labeled macrophages (green) in the proximity of a PS₁₀ microsphere (blue), between 4 to 5 hours after injection into a 3 days old embryo. Scale bar = 100 μ m; f) The blue fluorescent image of e) combined with the corresponding bright field image. The fluorescent images are part of a series of time laps images shown in Supporting Information Figure S1.

different for p values < 0.05. Of embryos that died during experiments, the measurements obtained on the days they were still live were included in statistical analysis.

RESULTS

Characterization of fluorescent microspheres

The size distribution of PCL₁₅ microspheres was 15.4 ± 1.6 μ m. According to the product information, PS₁₀ and PS₁₅ microspheres had very narrow size distributions of 9.9 ± 0.12 μ m and 15.4 ± 0.07 μ m, respectively. Scanning electron microscopic analysis confirmed the sizes of these three types of

microspheres [Fig. 2(c)]. All three types of microspheres were fluorescent owing to encapsulation of blue fluorescent dyes. PCL₁₅ microspheres were less bright than the two types of PS microspheres [Fig. 2(b)].

Cell interaction with microspheres in zebrafish embryos shortly after injection

To investigate whether PCL₁₅ microspheres provoke inflammatory cell responses in zebrafish embryos shortly after injection, we studied the migration of fluorescent protein-expressing macrophages (red) and neutrophils (green) between 1 to 2 hours post injection (hpi) of these microspheres into embryos of the zebrafish Tg line (fms:mCherry x mpo:eGFP) at 2 days post fertilization (dpf) [Fig. 3(a,b), Supporting Information Video S1]. The neutrophils apparently were rapidly attracted as they had already accumulated at the injection site at 1 hpi. Only a few macrophages were initially observed at the injection site, but their numbers increased between 1 and 2 hpi.

Neutrophil migration in response to an injected PS₁₀ microsphere was studied at 2 dpf using the zebrafish Tg line with only neutrophils fluorescently tagged (mpo:eGFP) [Fig. 3(c,d)]. A few neutrophils had already arrived in the vicinity of the microsphere within 1 hpi. More neutrophils subsequently were attracted to the injection site between 1 and 2 hpi (Supporting Information Video S2). Several neutrophils repeatedly moved toward and away from the PS₁₀ microsphere. Macrophage migration was separately studied after injection of a PS₁₀ microsphere at 3 dpf, using the zebrafish Tg line with only macrophages fluorescently tagged (Mpeg:Kaede). Between 4 to 5 hpi [Fig. 3(e,f), Supporting Information Fig. S1] a large number of macrophages accumulated in the muscle tissue in the proximity of the microsphere injected.

Quantification of cell migration toward injected PS₁₀ microspheres

We quantified the neutrophil and macrophage infiltration at the injection site in zebrafish embryos in response to injection of PS₁₀ microspheres or to PVP solution (Figs. 4 and 5). Nontreated embryos were used as controls. In the nontreated group, no accumulation of neutrophils or macrophages patrolling the tail tissue of embryos was observed during the entire experiment. This validated the use of the recorded fluorescence in this group as background levels.

Injection of PVP solution, the carrier of the microspheres, led to maximum levels of neutrophil infiltration at 5 hours post injection (hpi). The neutrophil infiltration strongly decreased at 1 day post injection (dpi) and further decreased to near background levels at 2 dpi, to reach background levels at 3 and 4 dpi (Figs. 4 and 5). Injection of PVP solution also induced a significant macrophage infiltration at 5 hpi, which increased to maximum levels at 1 dpi, and then gradually decreased to low levels but remained significantly elevated until the end of the experiment.

After injection of PS₁₀ microspheres the observed order of infiltration of neutrophils and macrophages was similar as triggered by injection of the carrier PVP solution. However, at

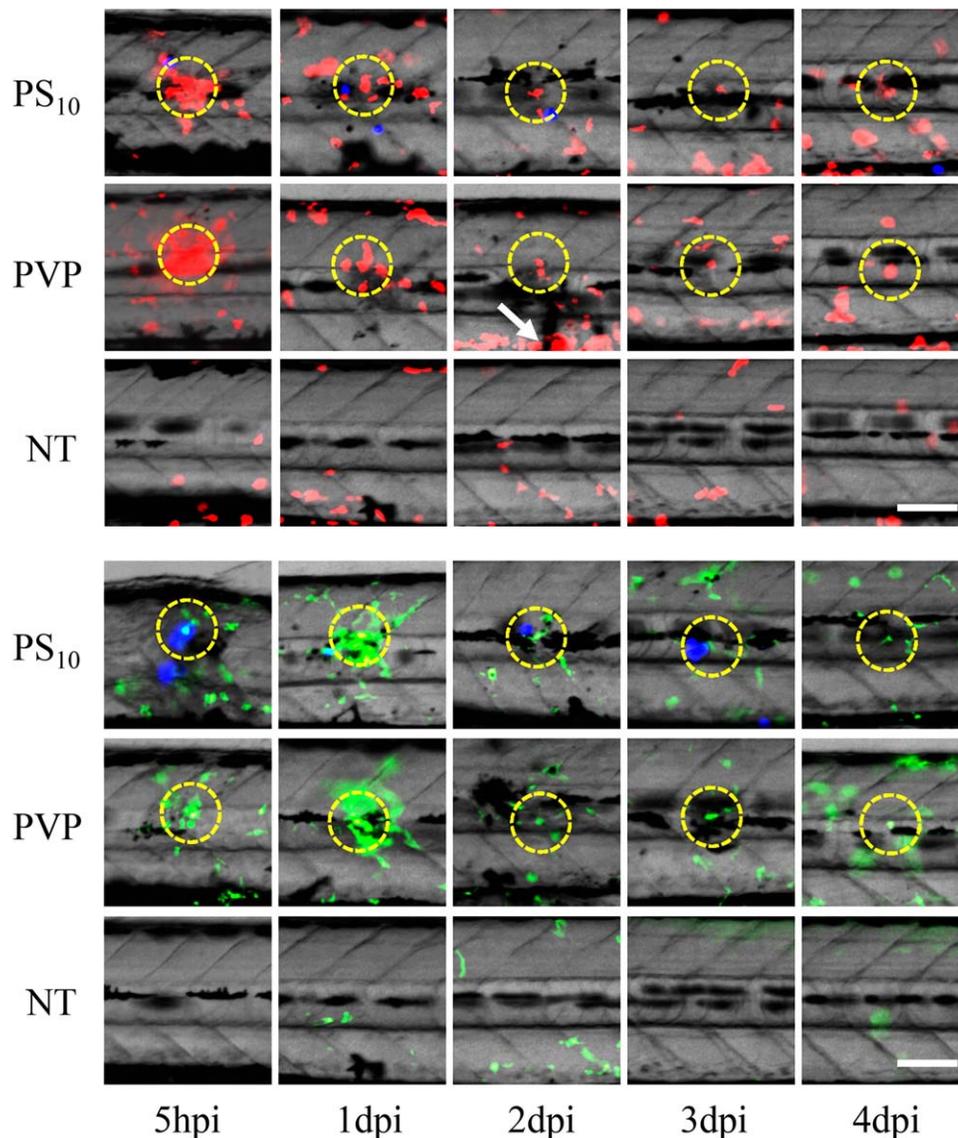


FIGURE 4. Neutrophil (red, top 3 rows) and macrophage (green, bottom 3 rows) infiltration in response to injected PS₁₀ microspheres or the carrier PVP solution alone, compared to background levels in nontreated embryos (NT), from 5 hours to 4 days post injection. Injections were performed at 3 days post fertilization. The yellow circles (100 μm in diameter) indicate the standardized area of fluorescence measurement. The arrow indicates circulating neutrophils. Scale bar = 100 μm .

5 hpi the level of infiltration of both cell types in the PVP solution group was higher than in the PS₁₀ group (Figs. 4 and 5).

Quantification of cell migration toward injected PS₁₅ and PCL₁₅ microspheres

To evaluate the cell infiltration in response to different microspheres, the neutrophil and macrophage infiltration triggered by injected PS₁₅ and PCL₁₅ microspheres was compared (Figs. 6 and 7). Injection of PS₁₅ or PCL₁₅ microspheres as well as of PVP alone caused a rapid neutrophil and subsequent macrophage infiltration. The maximum levels of neutrophil infiltration were recorded at 5 hpi. Levels were still strongly elevated at 1 dpi but decreased to near background levels at 2 dpi. At 1 dpi significantly higher levels of neutrophil infiltration were observed around PCL₁₅

than around PS₁₅. In all groups macrophages infiltrated later than neutrophils, reaching their maximum levels at 1 dpi. In the PCL₁₅ group, levels of macrophage infiltration remained higher than in the PS₁₅ group at 2 and 3 dpi. At 4 dpi macrophage levels in all groups had returned to background levels.

DISCUSSION

In humans as well as in animal models, the inflammatory cell response to inserted or implanted biomaterials is characterized by an initial rapid infiltration of neutrophils, followed by macrophages.^{1,26-28} To assess such cellular responses to biomaterials in a rapid *in vivo* assay we developed a zebrafish embryo model, making full use of the possibility to monitor cell infiltration in response to injected

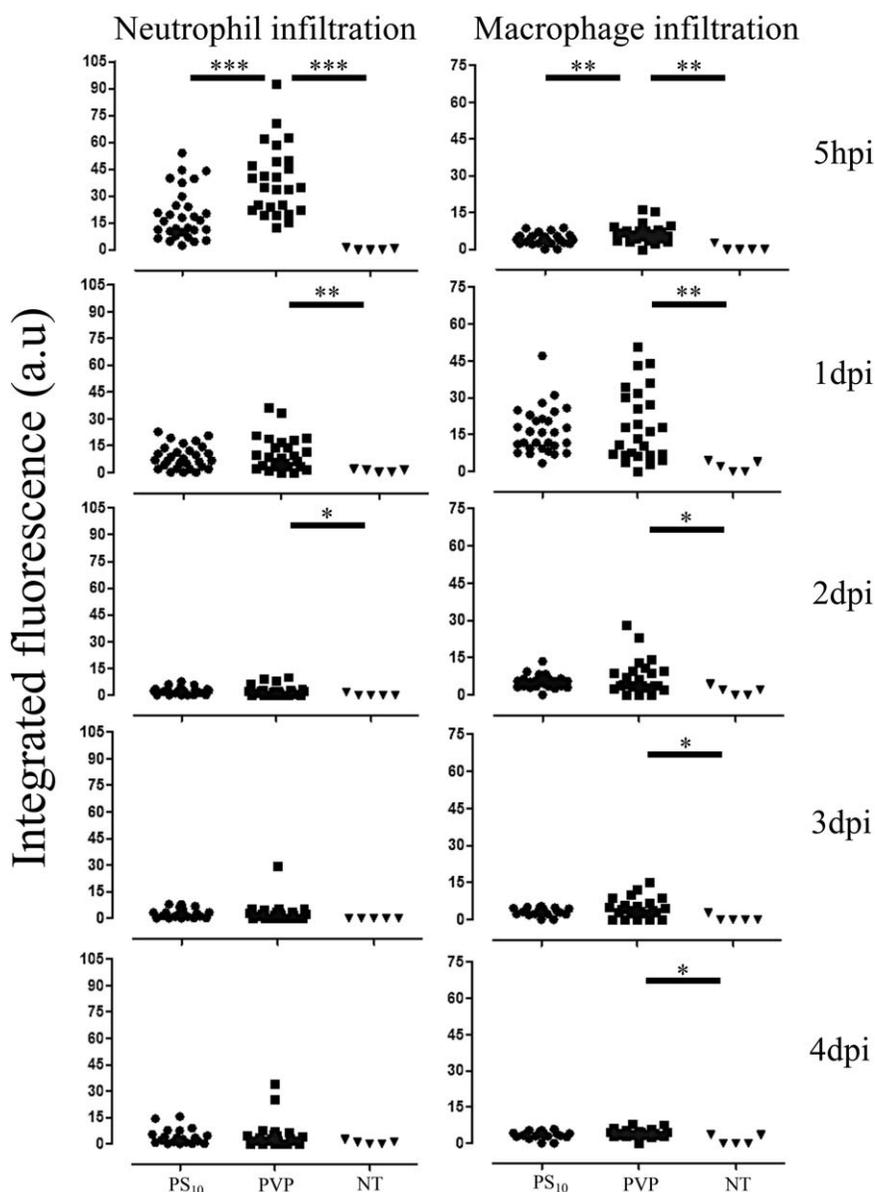


FIGURE 5. Quantification of neutrophil and macrophage infiltration in response to injected PS₁₀ microspheres or to injection of PVP solution in individual zebrafish embryos from 5 hours to 4 days post injection. Injections were performed at 3 days post fertilization. The infiltration was quantified as the integrated fluorescence (arbitrary units) of DsRed protein—expressing neutrophils and Kaede protein—expressing macrophages in the standardized area of measurement (yellow circles, Fig. 4). PS₁₀, embryos injected with PS₁₀ microspheres using 4% PVP solution as carrier; PVP, embryos injected with 4% PVP solution; NT, nontreated control embryos. Differences between pairs of groups (PVP vs. NT, PS₁₀ vs. PVP) were analyzed by the Mann Whitney test; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. During the experiment the number of embryos in the PS₁₀ group and in the PVP group decreased from 30 to 20 and from 26 to 22, respectively. The number of embryos in NT group remained at 5.

microspheres in real time, using fluorescence microscopy and semi-automated quantification of infiltration levels. Zebrafish embryos have been reported to possess a sophisticated innate immune system which is considered highly similar to their mammalian counterparts, particularly in the following aspects such as types of innate immune cells present and their functionalities (for example, phagocytosis of cell debris or microbes), expression of cytokines, and chemokines as well as signal transduction systems for cell recruitment and migration, and sensing of danger molecules. The similarities in the (innate) immune system of zebrafish

embryos and mammals have been summarized in several excellent reviews.^{9,16-18} In the present study, we observed the same order of cell infiltration in response to implanted biomaterials in the zebrafish embryo model as was reported in studies using mammalian models (for example, mice).²⁶⁻²⁹ In addition, the residence time of macrophages in response to injected microspheres in zebrafish embryos was similar to the residence time of macrophages in response to 1 μm PLGA microparticles injected into the subcutaneous tissue beneath an inserted biomaterial “window” replacing the skin of mice.²⁹ In both cases, the macrophage accumulation reached

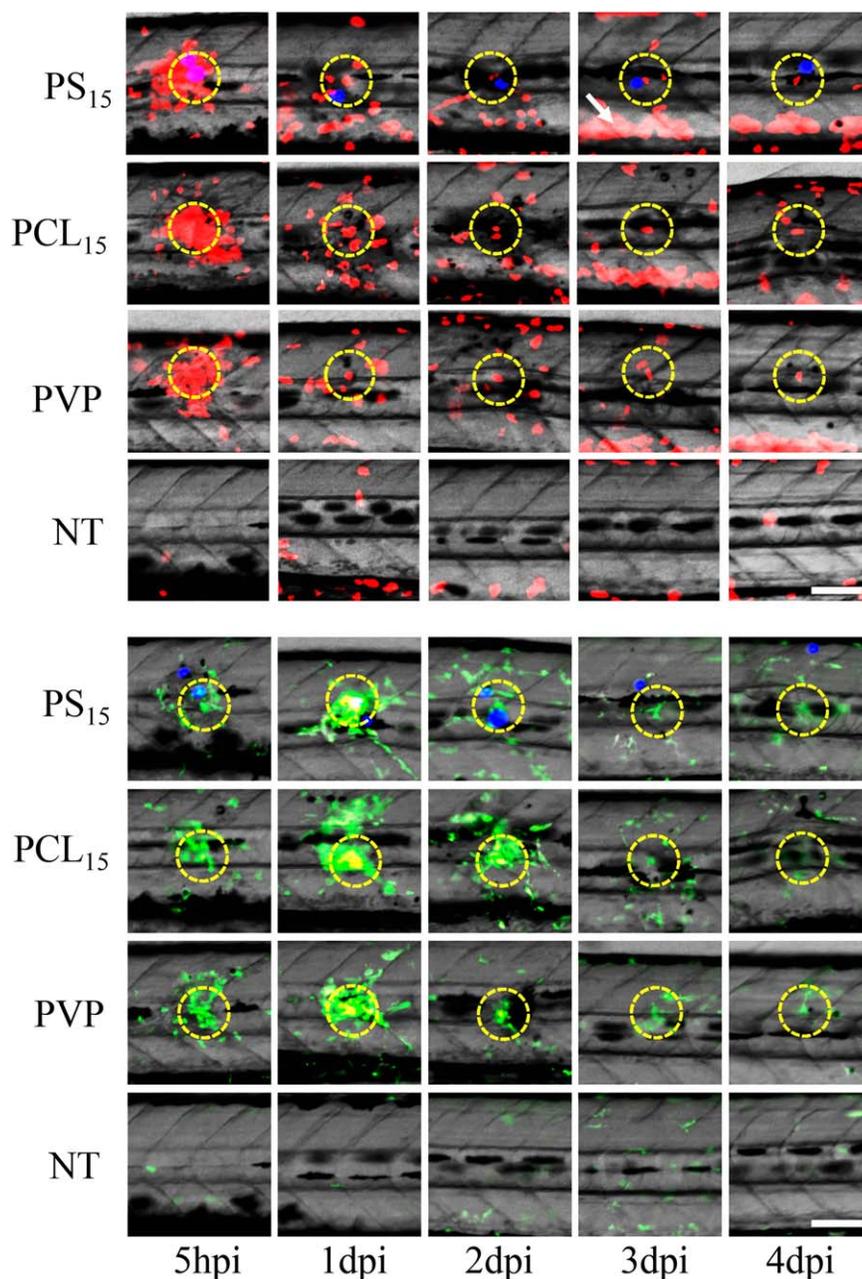


FIGURE 6. Neutrophil (red, top three rows) and macrophage (green, bottom three rows) infiltration in response to injected PS₁₅ microspheres, PCL₁₅ microspheres or the carrier PVP solution alone, compared to background levels in nontreated embryos (NT), from 5 hours to 4 days post injection. Injections were performed at 3 days post fertilization. The yellow circles indicate the standardized area of fluorescence measurement (100 μm in diameter). The arrow indicates circulating neutrophils. Scale bar = 100 μm .

maximal levels at 2 days post injection (dpi) and decreased to control levels at 4 dpi. Although the models are different, the similar timing of the macrophage response to injected biomaterials suggests that the zebrafish embryo model is reliable for assessing biomaterial associated inflammatory cell responses.

A variety of material properties such as chemical composition, shape, size, porosity, surface chemistry, and morphology may influence the extent of inflammatory cell responses to biomaterials.^{1,2,27,30} To assess the applicability of the zebrafish embryo model for analyzing such cellular responses, we

compared the cell infiltration provoked by microspheres differing in particle size (PS₁₀ and PS₁₅) and in chemical composition (PS₁₅ and PCL₁₅). PVP solution (4% w/v) is needed to keep the microspheres dispersed for injection into zebrafish embryos, therefore, we used injection of PVP solution as control. Although an inflammatory response was provoked by the injection of PVP solution, this did not preclude detection of inflammatory cell responses to the microspheres in the zebrafish embryo model. Because of the small size of the zebrafish embryos, we chose 15 μm as the maximal microsphere size, as it is not too large for the embryos and still allows efficient

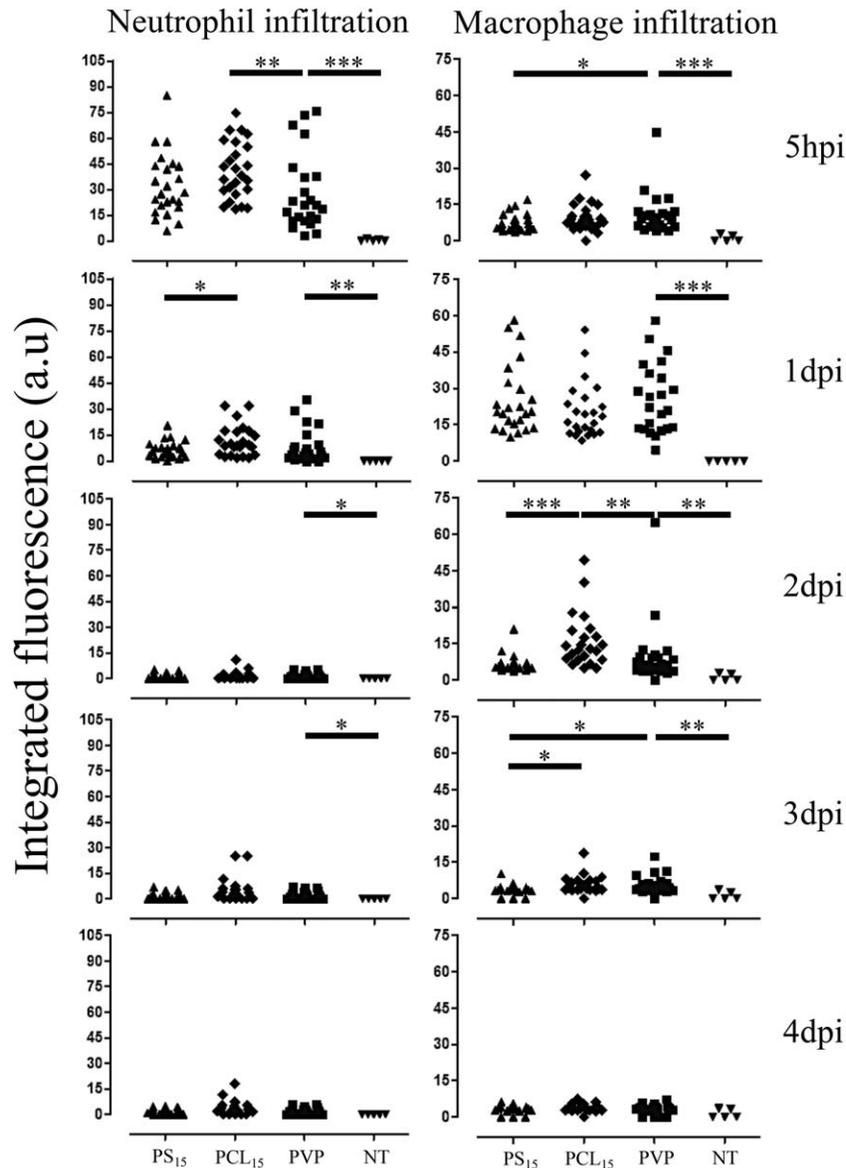


FIGURE 7. Quantification of neutrophil and macrophage infiltration in response to injected PS₁₅, PCL₁₅ or injection of PVP solution in individual zebrafish embryos from 5 hours to 4 days post injection. Injections were performed at 3 days post fertilization. The infiltration was recorded as the integrated fluorescence (arbitrary units) of DsRed protein—expressing neutrophils or Kaede protein—expressing macrophages in the standardized area of measurement (yellow circles, Fig. 6). PS₁₅, embryos injected with PS₁₅ microspheres using 4% PVP solution as carrier; PCL₁₅, embryos injected with PCL₁₅ microspheres using 4% PVP solution as carrier; PVP, embryos injected with 4% PVP solution; NT, nontreated controls. Differences between pairs of groups (PVP vs. NT, PCL₁₅ vs. PVP, PS₁₅ vs. PVP, PCL₁₅ vs. PS₁₅) were analyzed by the Mann Whitney test; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. During the experiment the number of embryos in the PS₁₅ group and in the PCL₁₅ group decreased from 24 to 23 and from 25 to 23, respectively. The number in the PVP group and in the NT group remained at 23 and 5, respectively.

injection. Moreover, we aimed to study the response to non-phagocytosed materials, since this is relevant for the response to implanted medical devices. *In vitro*^{31,32} and *in vivo* studies^{27,33} have shown that phagocytic cells of mouse, rabbit and human rarely phagocytose microspheres larger than 10 μm , which is why we chose 10 μm as the smallest microsphere size for our model. Indeed, in real time fluorescence microscopy of injected zebrafish embryos, we did not observe phagocytosis of the injected microspheres, neither by macrophages nor neutrophils during the daily observation periods. However, occasional phagocytosis cannot be ruled out since no

continuous observation was performed. More frequent observation periods (two times per day) may be considered for further studies.

PS₁₀ and PS₁₅ microspheres provoked similar levels of differences in the infiltration of neutrophils and macrophages in comparison to the responses provoked by injection of the carrier PVP solution alone. This indicates that the difference in particle size (5 μm) had no influence on cell infiltration in the present study. Effects of particle size on cell infiltration have been reported in mouse and rabbit models, but in these models the size differences of the

microspheres were much larger (at least 25 μm).^{33,34} Such effects might also be observed in zebrafish embryos when microspheres with larger size differences would be used.

Zebrafish embryos showed differences in the levels of inflammatory cell responses to microspheres of different composition. PCL₁₅ microspheres induced higher levels of infiltration of neutrophils at 1 day, and of macrophages at 2 as well as 3 days post injection, than PS₁₅ microspheres did. Microspheres of either of these two types of materials have been shown to provoke infiltration of inflammatory cells after injection into mice, rats or rabbits,^{27,34,35} but to the best of our knowledge these materials have never been tested side by side *in vivo*. The finding that the zebrafish embryo model revealed differences in cell infiltration levels induced by these materials, even though they both are biocompatible, indicates a high sensitivity of our testing system to detect differences in inflammatory characteristics of materials. Therefore, the zebrafish embryo model is expected to uncover possibly stronger differences in induction of cellular responses between other materials, allowing the discrimination of more inflammatory from less inflammatory materials. The molecular mechanisms behind these differences in inflammatory cell responses to PS₁₅ and PCL₁₅ are the subject of our ongoing studies. The zebrafish embryo model allows advanced analysis methods for inflammatory cell responses, such as *in vivo* tracking of single cell migration¹⁴ and high throughput transcriptome analysis of zebrafish genes encoding potentially important cytokines and chemokines (for example, IL-1 β).³⁶ Such analyses may reveal potential biomarkers which can be used to identify novel biomaterials with desired response induction.

For all types of microspheres, the resolution of macrophage responses rapidly occurred at 4 dpi in the present study. It is well established that the ratio between the two subsets of macrophages, namely pro-inflammatory M1 and anti-inflammatory M2 cells, is crucial for the resolution of inflammation.⁴ Interestingly, zebrafish embryos have been used to develop the first *in vivo* model allowing real-time monitoring of macrophage polarization, utilizing a new transgenic zebrafish line expressing distinct fluorescent proteins in M1 and M2 cells.³⁷ Polarization of macrophages into M1 and M2-like subtypes of the embryos of this zebrafish line has been shown to occur during the inflammation and resolution phase. Hence, this novel transgenic zebrafish line will offer the opportunity to study macrophage polarization in presence of biomaterials during different inflammation phases *in vivo*. Of note, in addition to innate immune cells, adaptive immune cells have also been reported to play a role in the host response to particular biomaterials and may have interaction with macrophages, particularly during chronic inflammation.³⁸ However, the adaptive immune system of zebrafish is not fully functional until 4 weeks post fertilization,^{7,16,20} so the influence of adaptive immunity in the model is expected to be very limited, and would need to be studied in more mature embryos.³⁹

The zebrafish embryo model offers the unique possibility to develop high throughput *in vivo* models for testing

biomaterials, required to complement the successful development of high throughput synthesis of biomaterials differing in chemistry and/or topography, and to complement *in vitro* analysis of induced cell behavior.^{40–42} The model is amendable to development of a high throughput screening system, that is, by using advanced robotic injection and imaging techniques.²⁰ The ImageJ plugin ObjectJ project file “Zebrafish Immunotest” developed in the present study has shown its value for analysis of medium-large image sets. It is also suitable for high throughput analysis and available through open access, a much desired characteristic for novel image analysis software.^{43,44} Combined with established methods of *in vitro* cytotoxicity screening, and supported by the possibilities of high throughput *in vivo* analysis, our zebrafish embryo model can help guide the selection of (novel) biomaterials with desired *in vivo* cellular response characteristics at an early stage of their development.

CONCLUSIONS

We have developed a zebrafish embryo model as a novel *in vivo* system with potential for rapid and semi-automated quantitative analysis of early inflammatory cell responses to injected microspheres. The observed inflammatory cell responses were very similar to those observed in mammalian animal models. Difference in size of microspheres (in the injectable size range; 10 and 15 μm in diameter) did not influence the elicited cellular responses. However, the difference in chemical composition between PCL and PS microspheres had significant impact on the elicited cellular responses. Our study therefore shows that zebrafish embryos are sufficiently sensitive to discover differences in the inflammatory cell response to biomaterials with different physicochemical characteristics. For future work, this embryo model can be developed into a high throughput system, complementing *in vitro* cytotoxicity testing for the efficient screening of (novel) biomaterials at an early stage of their development.

ACKNOWLEDGMENTS

We would like to thank Henk van Veen of the Electron Microscopy Center Amsterdam (EMCA) and Wim van Est from the Academic Medical Centre for help with the scanning electron microscopy and assisting in figure preparation, respectively, and Gert J. Veldhuis and his colleagues from Nanomi B.V (Oldenzaal, The Netherlands) for their help with microsphere preparation, and ZF-Screens B.V (Leiden, The Netherlands) for support in zebrafish embryo technology. This study is part of IBIZA project of the Biomedical Materials program, co-funded by the Dutch Ministry of Economic Affairs.

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