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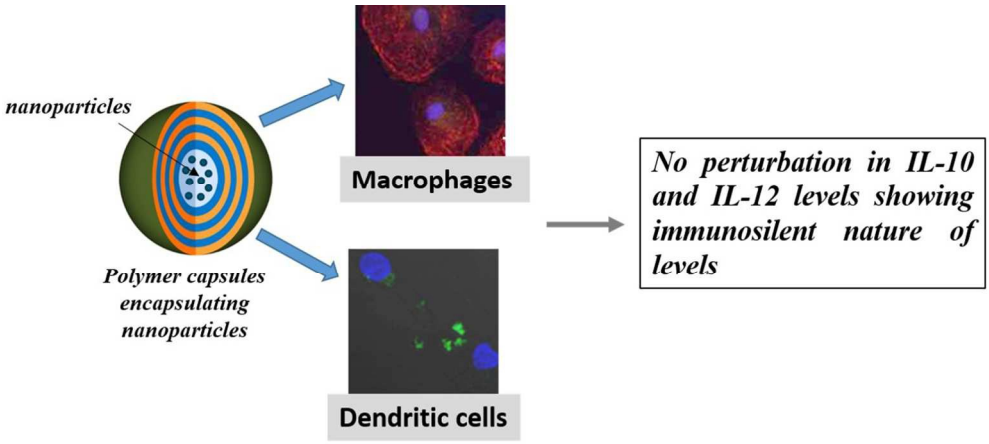

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Immuno-silent Polymer Capsules Encapsulating Nanoparticles for Bioimaging Applications

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Abstract

PEGylated polymer capsules encapsulating $LaVO_4:Tb^{3+}$, $GdVO_4:Tb^{3+}$, $Gd_2O_3:Tb^{3+}$, $GdF_3:Tb^{3+}$, $YVO_4:Tb^{3+}$ and iron oxide nanoparticles are promising new fluorescence, magnetic and magnetofluorescence imaging agents. Recently, we have reported the in vitro and in vivo level toxicity profile which shows the non-toxic nature of polymer capsules encapsulating nanoparticles. However, prior to clinical use, it is essential to ensure that these agents are unlikely to activate immune responses. Herein, we investigated the immuno-compatibility of polymer capsules with dendritic cells (DC) and macrophages (MO), major antigen presenting cell (APC) subsets required for activation of innate and adaptive immunity. Capsules were efficiently internalized by both DC and MO *in vitro*. Importantly, despite the presence of intracellular capsules, there was no significant impact on the viability of cells. We studied the impact of different capsules on the cytokine profile of DC and MO, known to be important for the polarization of T-cell immunity. None of the capsules elicited change in cytokine secretion from DC. Furthermore, capsules did not alter the polarization of either M1 or M2 MO subsets as determined by the balance of IL-12 and IL-10 secretion. These data support the notion that PEGylated polymer capsules loaded with nanoparticles have the potential to remain immunologically silent as they do not activate APC and neither do they hinder the response of DC or MO to pathogen activating signals. These systems, therefore, exhibit promising characteristics for bioimaging applications.

KEYWORDS: *PEGylated polymer capsules, M1 and M2 macrophages, dendritic cells, immune response*

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1.Introduction

The development of nanobioimaging agents possessing enhanced contrast, immunocompatibility, biocompatibility, increased circulation time in blood and proper clearance from body is a propelling research area of nanomedicine. Recently, various nanocontrast agents (e.g. gold, superparamagnetic iron oxide, gadolinium-based nanoparticle) including multimodal imaging agents (e.g. magentofluorescence, X-ray contrast agent with fluorescence meoity/MRI ability) are being explored for bioimaging applications to obtain precise information from the target site. In order to apply these materials at the clinical phase, they should be investigated for following important biological studies: *in vitro* toxicity,[1] i*n vivo* biodistribution/toxicity[2-4] and immune-toxicity.[5] Though most of the nanocontrast agents have been tested for *in vitro* cytotoxicity and to some extent, *in vivo* biodistribution, their immunogenicity studies are either rarely explored or they possess immnuotoxicity. We note that there are some reports available on the investigation of generation of reactive oxygen species (ROS) which is also an indicator of immune toxicity.[6] In summary, it is vital to explore the immune response of nanoparticles along with *in vitro* and *in vivo* toxicity studies.

Immune responses from the body are one of the complex biological mechanisms comprising of diverse signaling pathways. Once a foreign object (e.g. nanocontrast agents) enters the body, it is recognized as "non-self" by antigen presenting cells (APCs), such as macrophages and dendritic cells which trigger immune responses. Thus, it is essential to understand the immune response of newly developed nanocontrast agent, in addition to *in vitro* and *in vivo* tests. Two types of immune responses-innate and adaptive immunity are generated in our body by immune cells. Innate immunity is non-specific which is produced by cells including dendritic

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cells, macrophages, natural killer cells, granulocytes (basophils, neutrophils, eosinophils), mast cells etc. whereas, adaptive immunity is highly specific which is produced by B and T cells. The APC constantly travel through the tissues scavenging for antigens such as bacteria, viruses or foreign bodies.[7] Upon antigen recognition APC starts secreting cytokines and travel to regional lymph node in order to activate T cell responses. In addition to activating T-cells, APC polarize their responses towards inflammation for example, through secretion of IL-12 which stimulate the generation of Th1 or tolerance through secretion of IL-10 which promote regulatory T-cells induction (Treg).[8-12] Therefore, various cytokines (for example, IL-12, IL-10, IL-6, IL-23 etc.) secreted by the APCs alert other cells of adaptive immunity in defense system for triggering specific immune response, for entry of foreign particle thereby creating a complete cluster of defense. Since, cytokines are the primary molecules of immune response, the evaluation of cytokine levels are used as biomarkers for understanding immune modulation by nanomaterials.[13-15]Herein, we investigate immunological response of set of nanoparticles by monitoring cytokines secreted by APCs. In this regard, we investigated the response of healthy human monocyte derived dendritic cells (immature, mature) and macrophages (M1, M2) to novel candidate capsules containing a range of contrast agents.

Recently, we developed $LaVO_4:Tb^{3+}$, $YVO_4:Tb^{3+}$, $GdVO_4:Tb^{3+}$, $Gd_2O_3:Tb^{3+}$, $GdF_3:Tb^{3+}$ and iron oxide nanoparticles encapsulated polymer capsules for bioimaging applications.[16] These nanoparticles-encapsulated polymer capsules possess the following advantages: (a) biocompatibility, (b) various nanoparticles can be encapsulated via single approach, (c) targeted delivery of nanoparticles by antibody modification of capsules, (d) can be converted into theranostic vehicle by incorporation of drug molecules, and (e) single internalization mechanism of nanoparticles in different cells, (f) $LaVO_4:Tb^{3+}$ and $YVO_4:Tb^{3+}$ nanoparticles exhibit

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fluorescence for optical imaging[17, 18] whereas iron oxide can be employed for magnetic resonance (MR) imaging, (g) $GdVO_4:Tb^{3+}$, $Gd_2O_3:Tb^{3+}$, and $GdF_3:Tb^{3+}$ have been found to display both fluorescence (optical imaging) and MR imaging.[19, 20]In our recent reports, these polymer capsules encapsulating nanoparticles were demonstrated as biocompatible at both *in vitro* at *in vivo* levels.[16, 21]Since, they possess good imaging properties along with biocompatibility and efficient internalization, it is essential to study their immune response.

For this study, we have chosen macrophages (M1 and M2) and dendritic cells (mature and immature) derived from human blood of different donors. Enzyme linked immunosorbent assay (ELISA) has been carried out for detection of interleukins IL-10 and IL-12 which were marked to assess the onset of immune responses by APCs. We found that polymer capsules encapsulating nanoparticles are immune-silent as they neither increase (immune-stimulator) nor decrease (immune-suppressor) the levels of interleukins (biomarkers of APCs).

2. Materials and methods

2.1. Materials

Gadolinium nitrate hydrate ($Gd(NO_3)_3.xH_2O$) was prepared by nitrification of gadolinium oxide(Gd_2O_3), terbium (III) nitrate pentahydrate ($Tb(NO_3)_3.5H_2O$), yttrium nitrate tetrahydrate ($Y(NO_3)_3.4H_2O$), lanthanum nitrate hydrate ($LaNO_3.xH_2O$), polyethylene glycol (Mw. 10,000), poly(sodium 4-styrene-sulfonate) (Mw. 70,000), poly(allylamine hydrochloride) (Mw.56,000), bisamine (polyethylene glycol) Mw. 6,000, tetraethyl orthosilicate (TEOS), rhodamine B isothiocyante were purchased from Sigma-Aldrich with all the lanthanide salts of 99.9% purity and used without further purification. Polyethylene imine (PEI, Mw.70000 branched), Alfa

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Aesar, citric acid (Qualigens), liquor ammonia (about 24%), hydrofluoric acid (40%w/v), Merck; ammonium fluoride, sodium metavanadate ($NaVO_3$) from Lobachemie.

Buffy coats were obtained from National Blood Transfusion Service, whilst CD14+ monocyte isolation kit was obtained from Miltenyi Biotec. Histopaque 1.077, and RPMI were obtained from Sigma Aldrich. For cell uptake experiments, RITC-phalloidin, fluorescein isothiocyante (FITC) and fluorshield DAPI were obtained from Sigma Aldrich.

Dulbecco's modified eagle's medium (DMEM), trypsin- EDTA, penicillin-streptomycin antibiotic, (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT), were obtained from Sigma Aldrich and used without further purification. Fetal bovine serum was purchased from Gibco® and Cy 5.0 CellMask™ deep red plasma membrane stain (ex 649nm/em 666nm) was purchased from Invitrogen, India. Dimethylsulfoxide (DMSO) and formaldehyde were obtained from Merck Chemicals, India. rhGM-CSF was obtained from Peprotech (Rocky Hill, NJ,USA); rh M-CSF from ImmunoTools (Firesoythe, Germany); rhIL-4 and IFN-γ from R&D Systems Europe (Oxford, United Kingdom); Ultrapure LPS (Salmonella Minnesota LPS) was from InvivoGen (San Diego, CA,USA). Ultra-low attachment plates (Corning, NY, USA)

2.2. Generation of dendritic cells and macrophages

PBMCs were isolated from Buffy coats with Histopaque 1.077 by gradient centrifugation. CD14+ve monocytes were purified with anti-CD14 magnetic beads (Miltenyi). Monocyte derived DCs were generated as previously described.[22, 23] Briefly, positively selected CD14+ monocytes were cultured in DC medium (RPMI, 10%FBS, 1% sodium pyruvate) containing rhGM-CSF and rhIL-4 (both 1000 U/mL). Additional complete medium with cytokines was added on day 3-4.[24]

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Macrophages were generated from CD14+ve cells by culture in ultra-low attachment plates for 5-6 days in complete medium (RPMI, 10% FBS, 1% sodium pyruvate) with 20 U/mL GM-CSF for generation of M1 or 10 ng/mL M-CSF for generation of M2 phenotype. Additional complete medium with cytokines was added on day 4. The phenotype of cells was confirmed by flow cytometry and profiling of cytokines released on maturation. DC and M1 MO were activated with LPS (500 ng/mL) and IFN-γ 1000 U/mL) for 24h, M2 macrophages were activated with LPS (500 ng/mL) alone.

2.3. Cell uptake and MTT assay studies. For observing the internalization of polymer capsules, both mature DCs were seeded on sterile coverslips (50,000 cells per well) placed on 6-well plate. The capsules (25 capsules per cell) were incubated for 5 h with PEGylated PSS/PAH capsules encapsulating $LaVO_4:Tb^{3+}$, $YVO_4:Tb^{3+}$, $GdVO_4:Tb^{3+}$, $Gd_2O_3:Tb^{3+}$, $GdF_3:Tb^{3+}$ and iron oxide nanoparticles (synthesis procedure of polymer encapsulated nanoparticles are provided in supplementary information). The wells were washed with PBS to remove unbound capsules. The cells were fixed with formaldehyde (2%) for 20 min followed by washing twice with PBS. Coverslips were mounted on slides in Fluorshield DAPI to stain the nucleus. For cell uptake in IC-21 macrophages (mouse peritoneal cells), 10^5 capsules were seeded and rested overnight. Capsules (25 capsules per cell) were added to cells and incubated for 5 h. Unbound capsules were removed by washing twice with PBS prior to fixing with formaldehyde. Cell membranes were stained using deep red membrane dye (Cy5). MTT assay for quantifying cell viability was also carried out in same conditions as cell uptake studies.[25] UV-visible absorbance was recorded at 570 nm.

2.4. DC and MO phenotyping

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Flow cytometry was performed on MACS Quant flow cytometer (Miltenyi). For extracellular staining cells were stained with HLA-DR FITC, CD80 APC, CD86 Vio-blue (all Miltenyi), CD40 FITC, CD83 PE (BD Biosciences) for 30 min on ice. For CD68, cells were fixed and permeabilised using Inside Stain Kit (Miltenyi) and stained with CD68 FITC (Miltenyi). Flow cytometry experiments were analyzed in FlowJo software.

2.5. ELISA assay for quantification of cytokine levels

Immature DCs were seeded at 50,000 cells/well in DC media (RPMI 1640, 10% FCS, sodium pyruvate) and rested for 2 h. DCs were incubated with PEGylated polymer capsules encapsulating $LaVO_4:Tb^{3+}$, $YVO_4:Tb^{3+}$, $GdVO_4:Tb^{3+}$, $Gd_2O_3:Tb^{3+}$, $GdF_3:Tb^{3+}$ and iron oxide nanoparticles (25 capsules per cell) for 24 h. In order to get mature DCs, LPS/IFN-γ was added and incubated for 2 h. A similar protocol was followed for M1 and M2 macrophages. The secretion of IL12p70 and IL10 was performed by commercial human ELISA kit (eBioscience and R&D Duoset respectively). The sensitivity of IL-12p70 ELISA was 7.8 pg/mL and IL-10 was 31.2 pg/mL. Absorbance was read at 450 nm wavelengths using a spectrometer.

2.6. Characterizations

The morphology of the samples was acquired from SUPRA® Series Ultra High Resolution Field Emission-Scanning Electron Microscope FE-SEM (SEM). The formation of core-shell structure was visualized from Transmission Electron Microscope (TEM) images obtained using FEI Technai Twin microscope with high contrast and resolution at 20kV to 120kV. Internalization of polymer capsules in cells has been confirmed through images from laser scanning confocal

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microscope Carl Zeiss LSM 710 and LSM 810.UV-visible absorbance was recorded for MTT assay using Thermoscientific UV-Visible spectrophotometer.

2.7. Statistics

Statistical analysis was undertaken using Graphpad prism 5.0. ELISA assay data were plotted using mean ± SD and statistical significance determined with two-tailed *t*-test. In case of multiple data comparison, data is analyzed using one-way ANOVA test followed by Bonferroni post-test.

3. Results

3.1. Characteristics of nanoparticle-loaded capsules

Silica particles (~500 nm) were fabricated by modified Stöber's process.[26, 27] Lanthanide doped nanoparticles were coated over silica using a method adapted by Yu *et al*.[28] Synthesis of GdF_3:Tb^{3+} and iron oxide nanoparticles were carried out using PEGylated polymer capsules as microreactor.[25, 29] The formation of polymer capsules encapsulating $LaVO_4$:Tb^{3+}, $GdVO_4$:Tb^{3+}, Gd_2O_3:Tb^{3+}, GdF_3:Tb^{3+}, YVO_4:Tb^{3+} and iron oxide nanoparticles have been confirmed by performing transmission electron microscopy (TEM). TEM images (Figure 1) show contrast difference between polymer capsules and nanoparticles (size: ~45 nm) confirming the entrapment of nanoparticles inside capsules.[16] HRTEM images display lattice fringes showing crystalline nature of material.

3.2. Interaction of capsules with DC and MO

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Internalization of capsules was studied in DC, followed by quantification of interleukins to understand behavior of APCs on interaction with polymer capsules. In the case of cell uptake studies, CLSM images Figure 2 shows PEGylated polymer capsules internalized by DCs after 5 h incubation time. The internalization is confirmed by green emission arising from terbium ions (λ_{ex}=488 nm) doped in $LaVO_4/GdVO_4/Gd_2O_3/GdF_3/YVO_4$ nanoparticles and red emission from RITC-labeled PAH layer of capsules loaded with iron oxide. Since the number of macrophages obtained from human blood was insufficient for cell uptake and cytotoxicity assays, a peritoneal macrophages cell line (IC-21, murine) was substituted. Figure 3 represents internalization of polymer capsules encapsulating nanoparticles in IC-21 cells (revealed by green emission from terbium ions/FITC labeled PAH layer). Capsules were not observed to adversely impact the viability of any cells studied. Figure S1 represents MTT assay plot exhibiting biocompatibility of capsules with macrophages.

3.3. Capsules do not activate resting DC nor perturb DC activation

The purity and quality of generated DC was assessed by phenotypic analysis. In agreement with previous reports DC generated from CD14+ve monocytes in the presence of GM-CSF and IL-4 showed loss of CD14 expression, high HLA-DR and were positive for CD80, CD40, CD86 while CD83 expression was negative.[22, 23] Upon activation with LPS/IFN-γ for 24 h DC demonstrated typical signs of maturation by increase in HLA-DR, CD80, CD40, CD86 and gain of CD83 (Figure S2a,b).[30-34]

Resting DC and MO are very sensitive to a wide range of signals that indicate the presence of potential pathogens or damaged tissues. Therefore, novel imaging reagents would ideally not result in activation of resting APC. In order to test this, resting cells were exposed to capsules

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and their release of cytokines determined. Immature DC produced very little IL-10 and no IL-12. Upon exposure to LPS, mature DC (mDC) secreted markedly increased levels of both cytokines. Incubation of APCs with polymer capsules encapsulating $LaVO_4$, $GdVO_4$, Gd_2O_3, iron oxide and YVO_4 nanoparticles did not significantly modulate cytokine production from immature DC (Figure 4).Upon activation through pathogen receptors (e.g. TLR4), DC produce an array of cytokines that are involved in the polarization of subsequent immune responses. Therefore, we tested the impact of capsules on the maturation response of DC to the TLR4-agonist, LPS. The secretion of IL-12 and IL-10 by matured DC was not affected by the addition of nanoparticle-loaded capsules.

3.4. Capsules do not alter the polarization of M1 or M2 macrophages

Macrophages are broadly classified into M1 and M2 type based on their phenotype and cytokine expression. M1 was confirmed by positive staining with CD68. Both M1 and M2 macrophages expressed HLA-DR, CD86 and CD80 which increased upon activation. Expression of HLA-DR and CD86 was greater in resting and activated M1 compared to M2 macrophages. CD83 and CD40 were absent on immature and mature M2 but whilst absent on immature M1 they were expressed by mature M1 (Figure S3a,b).[30, 34]

Mature M1 macrophages produced high levels on IL-12 and low IL-10 while mature M2 macrophages preferentially secreted IL-10 whereas IL-12 levels were low. M1 macrophages incubated with polymer capsules encapsulating nanoparticles expressed similar levels of IL-12 and IL-10 after LPS activation compared to control (only cells) indicating that they are not altering the immune response of cells (Figures 5a and c). Similarly, M2 incubated with polymer capsules do not show altered expression levels of IL10 and IL-12 comparable to control (only

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cells) indicating immune compatibility of polymer capsules encapsulating nanoparticles (Figures 5c and d) in all.

4. Discussion

Synthesis of PEGylated polymer capsules encapsulating $YVO_4/GdVO_4/LaVO_4/Gd_2O_3:Ln^{3+}$ nanoparticles involves following steps: (a) fabrication of $YVO_4/GdVO_4/LaVO_4/Gd_2O_3:Ln^{3+}$ shell over silica to form a core-shell structure by sol-gel method, (b) deposition of PSS/PAH polyelectrolytes (LbL assembly) on core-shell particles, (c) PEGylation (stealth coating),[35] and (d) removal of silica core using buffer oxide etchant leading to formation of PEGylated polymer capsules encapsulating nanoparticles.

The polymer capsules were tested for its immunocompatibility by tracking the changes in interlukin secretions by APCs. Interleukins are also an indicator of reactive oxygen species (ROS) generation which leads to organ damage via inflammation.[6] Hanley *et al.* have reported ZnO nanoparticles-induced enhanced secretion of proinflammatory cytokines in polymorphic blood mononuclear cells (PBMCs) and monocytes.[36] In another report, gadolinium nanoparticles were found to exhibit minimal immunotoxicity which was also quantified by interleukin secretions from blood serum.[37]

We have demonstrated the *in vitro* biocompatibility and efficient internalization of PEGylated polymer capsules encapsulating nanoparticles in 7 different cell lines including HeLa, MCF-7, H460, A498, L929, Schwann cells and IC-21 MO. In addition, herein we have investigated the immunocompatibility of polymer capsules with human blood derived dendritic cells and macrophages. Dendritic cells were isolated from PBMCs as immature cells. Lipopolysaccharide

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(LPS), a major TLR-4 (Toll-like receptor) agonist was added to iDCs for artificially maturating them.[38] Upon maturation, DC secrete cytokines including IL-10 and 12 that modulate the activation of T-cells during their activation by DC. Our study focused on IL-12 and IL-10 for their role in Th1 and Th2/Treg responses respectively. PEGylated polymer capsules had no impact on IL-10 or IL-12 secretion (Figure 4).

Though in most of the reports, cytokine secretion has been studied in DC, it is equally important to explore it in macrophages as IL-10 and IL-12 secreted by them also influences immune response. Macrophages are classified as M1 (anti-tumor) and M2 (pro tumor) in which M1 suppresses tumor activity, promote Th1 responses whereas, M2 promote tumor growth through angiogenesis and anti-inflammatory effects.[12]M1 macrophages expressed higher levels of IL-12, in contrast to M2 macrophages which expressed higher levels of IL-10, though slight decrease in IL levels were observed with GdF_3 -loaded capsules which can be attributed to presence of Gd^{3+} ions on the surface of capsules.

It should be borne in mind that our study focused on the early events in terms of activation of antigen presenting cells. Future studies should address the potential for delayed immune events or indeed consequences that arise from repeated exposure to the capsules. Such events may include altered homing to draining lymph nodes, cross-presentation of antigens, or complex changes in gene expression. The silent nature of the tested capsules may mean it would be interesting to also investigate any impact on immune tolerance.

In summary, the present study demonstrated the immunological silence of the tested polymer capsules in both resting and activated DC and macrophages making them promising candidates for further work. IL-10 and IL-12 cytokine responses are very sensitive to confounding factors

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that activate or inhibit the function of these cells. Future studies will examine the effect of polymer capsules on specialized advanced functions of DC and MO including chemotaxis, antigen-presentation and cross-presentation.

5. Conclusions

PEGylated polymer capsules encapsulating $LaVO_4:Tb^{3+}$, $GdVO_4:Tb^{3+}$, $Gd_2O_3:Tb^{3+}$, $GdF_3:Tb^{3+}$ iron oxide nanoparticles were studied for their immune responses in dendritic cells (mature and immature) and macrophages (M1and M2). From cell uptake studies in dendritic cells and macrophages, it has been observed that capsules were internalized in the cells. ELISA assay performed to measure interleukins IL-10 and IL-12 levels, indicates comparable levels of secretion with the control. The capsules encapsulating nanoparticles are neither involved in immuosuppression or inflammatory response. The results conclude that PEGylated polymer capsules are not inducing any immune response confirming their safe applicability as bioimaging agents.

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Author Contributions

A.J., S.S., and P.P. designed the experiments. J.J., A.M. and P.B. performed experiments involving human blood derived primary cells. A.S. and J.J are involved in experiments with

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mouse peritoneal cavity derived macrophages. J.J and B.M. synthesized polymer encapsulated nanoparticles. A.J., S.S., and P.P. guided the work.

Notes

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View Article Online
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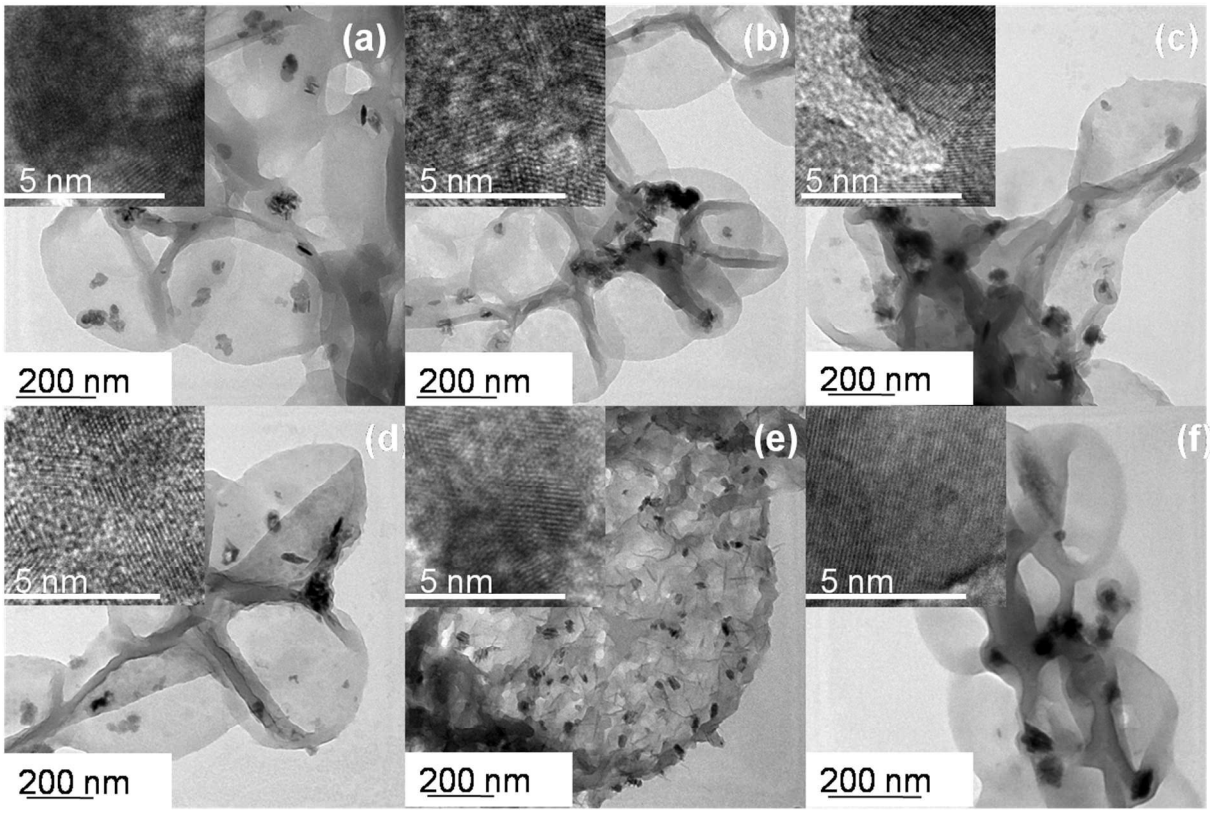

Figure 1. TEM images of PEGylated polymer capsules encapsulating: (a) $LaVO_4:Tb^{3+}$, (b) $YVO_4:Tb^{3+}$, (c) $GdF_3:Tb^{3+}$, (d) $GdVO_4:Tb^{3+}$, (e) iron oxide and (f) $Gd_2O_3:Tb^{3+}$ nanoparticles (Inset: HR-TEM images showing lattice fringes of respective nanoparticles).

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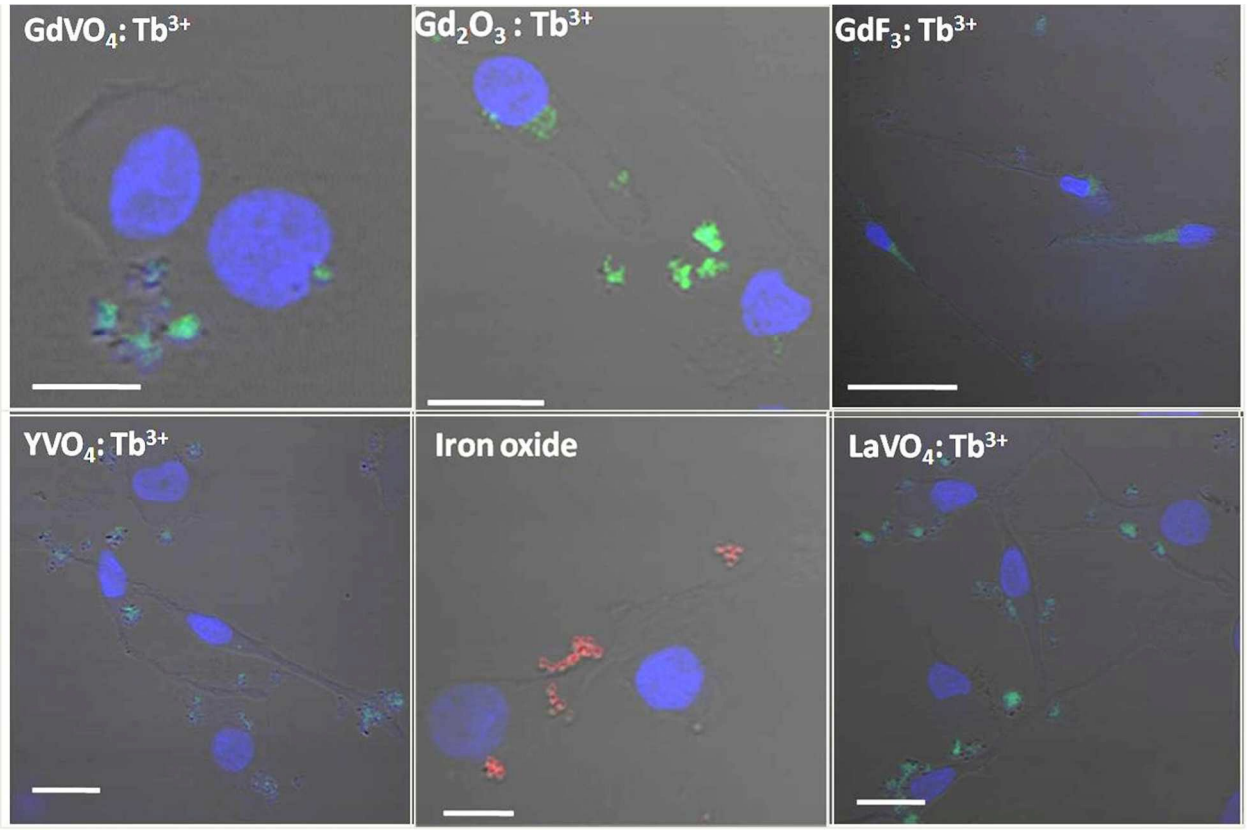

Figure 2. Confocal laser scanning microscopy images showing internalization of PEGylated polymer capsules encapsulating Gd_2O_3:Tb^{3+}, $GdVO_4$:Tb^{3+}, GdF_3:Tb^{3+} and iron oxide nanoparticles in mature dendritic cells (scale bar = 20 μm).

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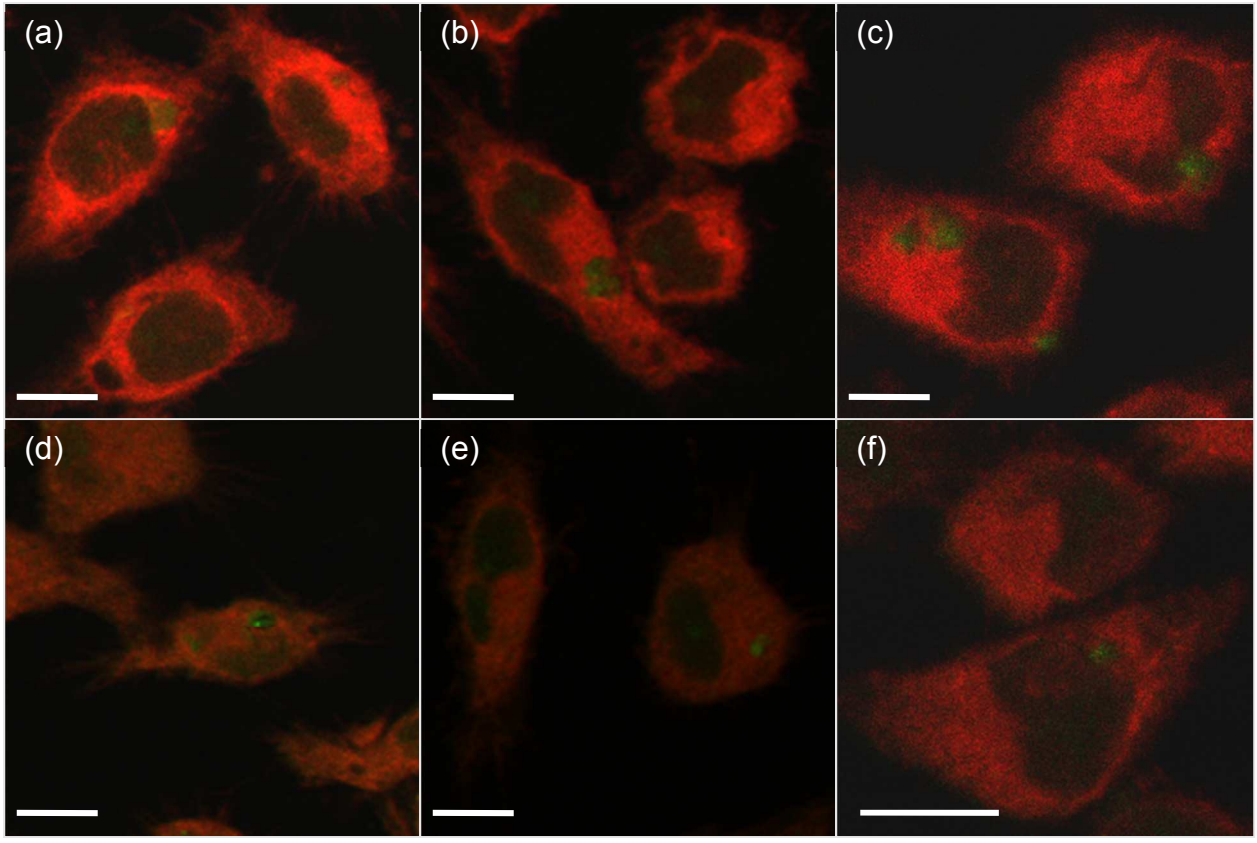

Figure 3. CLSM images showing internalization of PEGylated polymer capsules encapsulating nanoparticles in IC-21 cell line (mouse peritoneal macrophages): (a) $LaVO_4:Tb^{3+}$, (b) $YVO_4:Tb^{3+}$, (c) iron oxide (capsules labeled with FITC), (d) $GdVO_4:Tb^{3+}$, (e) $Gd_2O_3:Tb^{3+}$ and $GdF_3:Tb^{3+}$(scale bar = 20 μm).

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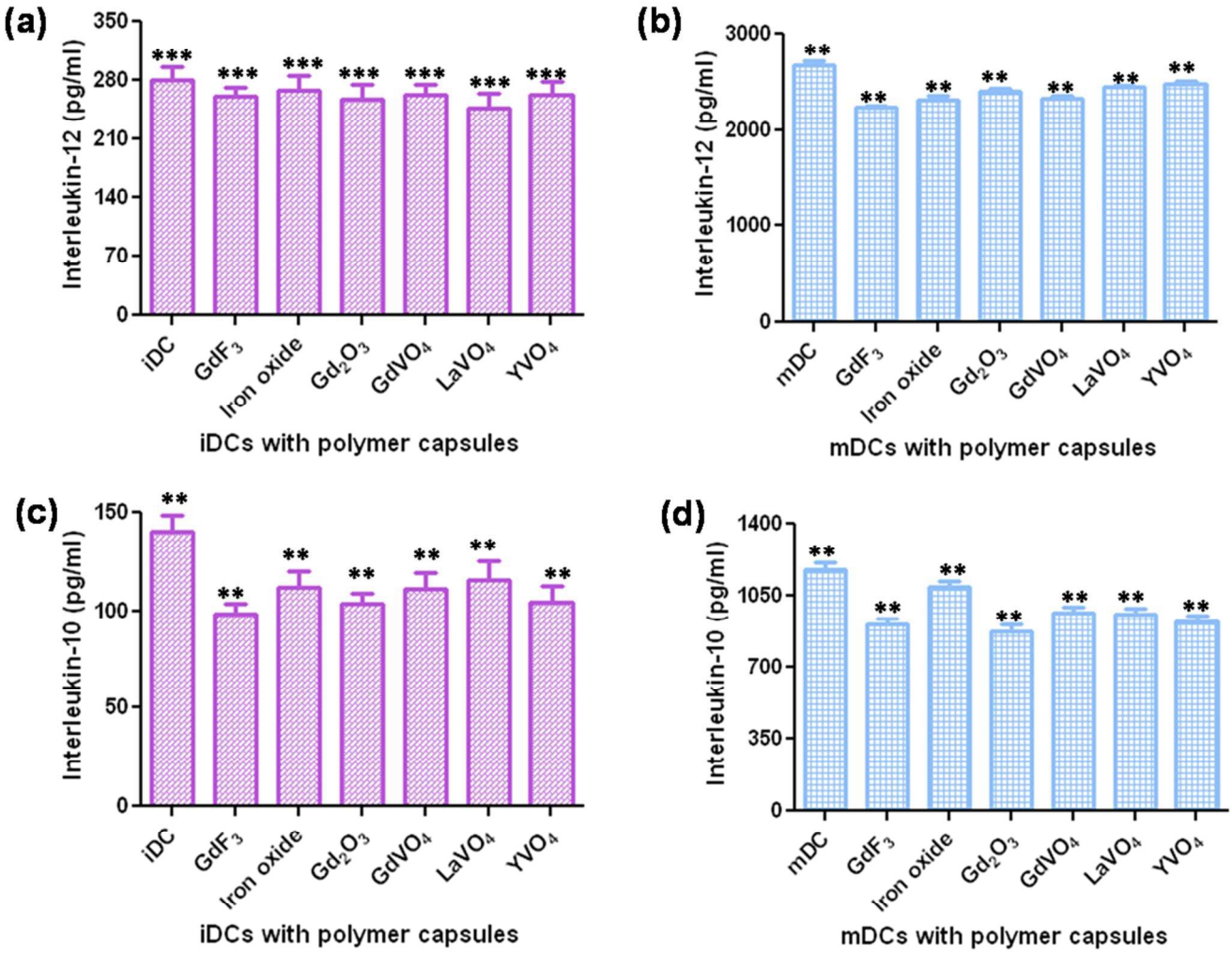

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Figure 4. ELISA assay plots of PEGylated polymer capsules encapsulating nanoparticles in immature and mature dendritic cells for detecting (a,b) IL-12 secretion (in pg/ml) and (c,d) IL-10 (in pg/ml) of four donors(*** signifies p<0.0001, ** signifies p<0.005).

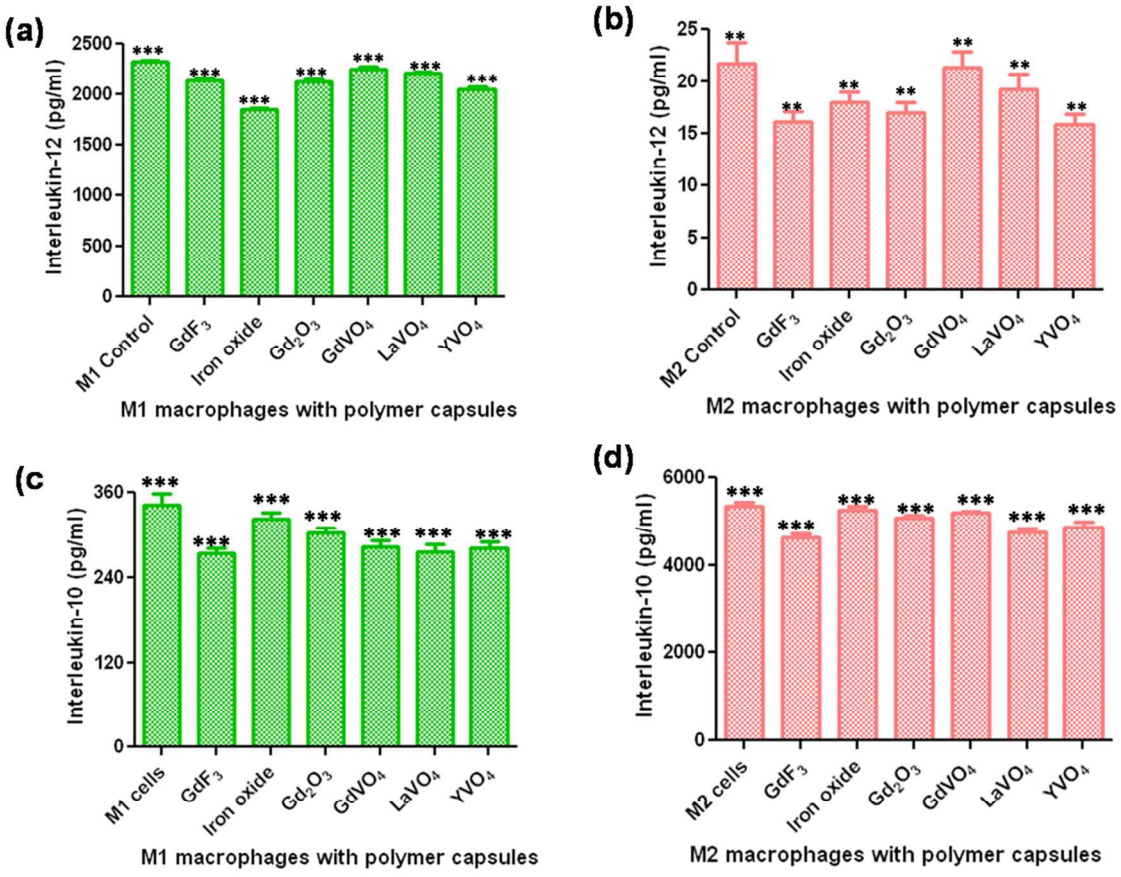

Figure 5. ELISA assay plots of PEGylated polymer capsules encapsulating nanoparticles in M1 and M2 macrophages for detecting (a,b) IL-12 secretion (in pg/ml) and (c,d) IL-10 (in pg/ml) of four donors (*** signifies p<0.0001, ** signifies p<0.005).

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