Evaluation of Biocompatibility and Release of Reactive Oxygen Species of Aluminum Oxide-Coated Materials

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ABSTRACT: Surface properties of biomaterials can strongly influence biomaterial–host interactions. For this reason, coating processes open a wide range of possibilities to modulate the fate of a biomaterial in the body. This study evaluates the effect of a coating material intended for drug delivery capsules on biocompatibility and the release of reactive oxygen species (ROS), that is, respiratory burst in macrophages that indicates acute inflammation. In parallel with a new approach to develop drug-delivery capsules by directly coating solid-state drug particles, in this study, glass slides and silicon nanoparticles (NPs) were coated with aluminum oxide (Al2O3) using atomic layer deposition. Different sizes of NPs (20 and 310 nm) were suspended at different concentrations (10, 100, and 1000 μg/mL) and were evaluated. The homogeneous coating of slides was proved using X-ray photoelectron spectroscopy, and the coating on NP was observed using transmission electron microscopy. Human dermal fibroblasts and human osteoblasts were able to proliferate on the coated slides and in the presence of a suspension of coated NPs (20 and 310 nm) at a low concentration (10 μg/mL). The macrophages released ROS only when in contact with NPs at a concentration of 1000 μg/mL, where the 20 nm NPs caused a higher release of ROS than the 310 nm NPs. This study shows that Al2O3 coatings do not affect the cells negatively and that the cell viability was compromised only when in contact with a high concentration (1000 μg/mL) of smaller (20 nm) NPs.

1. INTRODUCTION

Biomaterials have been used over decades with the principal aim to restore the function of tissues.1 Nowadays, biomaterials are used in a wide range of applications such as tissue engineering, medical imaging, diagnosis, and drug delivery.2 The interaction between a biomaterial and the host tissue is mainly determined by the physical properties of the biomaterials, such as surface, size, and mechanical properties.3 These properties in fact determine the application of the biomaterials and their fate in the host body.3,4 The surface properties of a biomaterial, which depend on its reactive sites and topography, determine the interaction with surrounding molecules, from small functional groups to proteins.4 These interactions are of prime importance because they will determine whether proteins adhere on a biomaterial with a functional state and further react with cell receptors.

To modify the surface properties of biomaterials, coating techniques have been used. Plasma spray, physical vapor deposition, and chemical vapor deposition are some of the most common techniques5 to perform inorganic, organic, and inorganic–organic coatings on inorganic materials.5,6 Atomic layer deposition (ALD) is a type of chemical vapor deposition technique that can be used to form nanometer thick coatings on three-dimensional samples with complex shapes. The ALD procedure consists of exposing the sample alternatively to gaseous precursors instead of having them present simultaneously in the reactor, as usually performed in chemical vapor deposition techniques. The process is repeated in cycles to grow a layer of substrate of desired nanometer thickness.6

A novel approach to produce drug-delivery systems (DDSs) consisting of solid-state drug particles directly coated with a ceramic material has been recently developed by Nanexa and trademarked as PharmaShell. The thickness of the coating, which can be controlled down to a monolayer of atoms, is designed to fulfill the desired drug-release profile. This DDS offers an opportunity for the pharmaceutical industry to develop controlled-release versions of established drugs with a high drug-to-weight ratio.

The use of nanoscale biomaterials, mainly used as DDSs, has increased in the last decade,7,8 raising the question of whether nanoparticles (NPs) are safe enough.8 NPs cannot be viewed as simple carriers because they play an active role in mediating
biological effects. The size of the NPs determines the circulation time and the interaction with inflammatory cells such as macrophages. Macrophages, which are distributed in several tissues to detect invading pathogens, may perform endocytosis of NPs and as well may trigger an acute inflammatory response. The acute inflammatory response mainly consists of the release of reactive oxygen species (ROS) aimed to destroy any pathogen or foreign body and the release of cyto- and chemokines that stimulate endothelial cells and fibroblasts to grow a vascularized tissue around the biomaterial. Several studies have shown how the cytotoxicity of NPs depends mainly on their size, shape, chemistry, and surface properties.

In this study, we aimed to evaluate the effect of aluminum oxide coatings on the growth of fibroblasts and osteoblasts and the release of ROS by macrophages. For this purpose, inert slides and NPs have been used instead of solid-state drug particles. The slides allowed us to determine the effect of the chemical nature of the coating, whereas the NPs allowed us to investigate the effect of the particle size.

2. RESULTS

2.1. Characterization of the Coated Slides Using X-ray Photoelectron Spectroscopy. Figure 1 shows a representative X-ray photoelectron spectroscopy (XPS) survey spectrum of an Al2O3-coated glass slide. Signals from aluminum, silicon, oxygen, and carbon were detected in all cases. The elemental composition and atomic ratio are given in Table 1. The O/Al atomic ratio was 2.3, whereas the O/(Al + C) atomic ratio was 1.6.

2.2. Characterization of the Coated NPs Using Transmission Electron Microscopy. NP suspensions were visualized using transmission electron microscopy (TEM). Figure 2 shows suspensions of NP310 and NP20. NP310 and NP20 formed agglomeration of ~20 nm NPs (Figure 2b) probably owing to their higher surface energy. Figure 2c shows a zoomed area of NP310, where the coating around the particle can be visualized and approximated to be 15–30 nm.

2.3. Cytotoxicity Assay. The cell viability on coated glass slides showed very similar trends for both cell lines used, human dermal fibroblasts (hDF) and human osteoblasts (Saos-2) (Figure 3). A similar fluorescence was determined for the coated samples and controls at day 1 (p = 0.763 and 0.292 for hDF and Saos-2, respectively), showing that the cells were able to proliferate equally well on the coated material as on the controls. Moreover, a statistically significant increase in fluorescence was observed for both cell lines on both materials (coated samples and controls) from day 1 to day 3 (p = 0 for all). The number of cells on day 3 was similar on the coated samples and controls (p = 0.621 and 0.766 for hDF and Saos-2, respectively), showing that a similar proliferation rate occurred on both surfaces. Both hDF and Saos-2 adhered and spread on the Al2O3-coated samples at 24 h (Figure 4).

The viability of cells in contact with a suspension (10, 100, and 1000 μg/mL) of coated NPs of different sizes (20 and 310 nm) was evaluated (Figure 5). Regarding hDF, at day 1, no significant difference (p = 1 for all cases) in the cell number was observed between the samples and the control. Cells cultured with any concentration of NP310, or cultured with NP20 at 10 and 100 μg/mL, increased significantly (p = 0) in cell number from day 1 to day 3. At day 3, only NP20 at 1000 μg/mL showed a significantly lower cell number than the control (p = 0.017). Although not statistically significant, NP310 at 1000 μg/mL and NP20 at 100 μg/mL caused a decrease in the cell number in comparison with the control. Similar results were observed for Saos-2, where only cells cultured with NP20 at 100 and 1000 μg/mL showed a significantly lower cell number than the control both at day 1 and day 3 (p = 0 for both concentrations and times). Cells cultured with NP310 at 1000 μg/mL also showed a significantly lower cell number (p = 0) than the control at day 3.

2.4. Release of ROS. Figure 6a shows luminescence signals when the macrophages were exposed to 1000 μg/mL NPs. Nonactivated cells and PMA-activated cells were used as negative and positive controls, respectively. The luminescence showed separated particles with a round morphology of ~300 nm in diameter (Figure 2a), whereas NP20 formed agglomerates of ~20 nm NPs (Figure 2b) probably owing to their higher surface energy. Figure 2c shows a zoomed area of NP310, where the coating around the particle can be visualized and approximated to be 15–30 nm.

Figure 1. XPS scan data for Al2O3-coated glass slides.

Figure 2. TEM representative images of the NP suspensions: (a) NP310 and (b) NP20. (c) Zoomed area of NP310.
signals are correlated with the amount of ROS released by cells, which is attributed to an acute inflammatory response. The activated cells released the highest amount of ROS, peaking at around 13 min. NP20 induced the cells to release a higher amount of ROS than that of NP310, both showing maximum peaks at around 17 min. Nonactivated cells did not cause any peak (Figure 6a). The macrophages exposed to 10 and 100 μg/mL of NPs showed a similar trend as the nonactivated cells (not included in Figure 6a for clarity). Figure 6b shows the values obtained by integrating the area under the luminescent curve and normalizing by the negative control. According to the integrated area, the release of ROS followed the order: +C > NP20 [1000] > NP310 [1000] > NP20 [10] and [100] ≈ NP310 at [10] and [100] ≈ −C.

3. DISCUSSION

A new approach to synthesize drugs with a high amount of active principle per weight consists of directly coating a drug particle with an inorganic material using the ALD technique (Figure 7). This unique DDS, trademarked as PharmaShell by Nanexa, offers a versatile way for generating carriers of any thickness as they can be directly fabricated on top of the drug particles. This technique could thus be revolutionary for pharmaceutical companies that are seeking new ways to produce more efficient products (with high drug/carrier mass ratio). However, coating drugs or biomaterials with inorganic compounds modifies their surface properties and has the potential to strongly influence their host interactions. This study evaluated the responses of two different cell types with respect to cytocompatibility and the release of ROS using inert slides and NPs coated with aluminum oxide.

The ALD conditions used are important to control the thickness of the coating. Under the conditions applied in our study, a thickness of 15−30 nm was formed on NP310 after applying 200 cycles, which correlates well with a growth rate of 0.11 nm per cycle described by Ott et al. The coating around NP310 particles (Figure 2c) was observed using TEM, thanks to the difference in electron density between silicon oxide and aluminum oxide, which creates a contrast between the coating and the particle. The TEM images observed in this study correlated well with the images displayed by a previous study that also used ALD to coat silicon oxide particles with aluminum oxide. In the case of NP20s, these NPs tended to agglomerate as shown in Figure 2b. The composition and homogeneity of the coating on glass slides were evaluated using XPS (Figure 1, Table 1). The coating was composed of atoms of aluminum and oxygen with a similar O/Al ratio in all replicate samples, proving a good homogeneity of the coating. The O/Al ratio was 2.2, a value significantly higher than the O/Al ratio of 1.5 that would be expected in the case of forming a stoichiometric aluminum oxide. The O/(Al + C) was 1.6, which suggested the presence of some C, probably in the form of methyl groups, from the initial reagent trimethyl aluminum (TMA), in accordance with the study of Kim et al. The chemical composition of the coating on the NPs was expected to be the same as the one determined on the slides because the same ALD principle was applied.

The cytotoxicity was evaluated using human cell lines from two different tissues because these potential DDS could come in contact with different parts of the body. hDF and Saos-2 were selected because they are commonly used to evaluate the biocompatibility of biomaterials. Moreover, it is known that NPs can lead to different cell fates depending on the cell type. Both hDF and Saos-2 cells were able to grow equally well on the aluminum oxide-coated slides as on the control material, that is, tissue-culture treated polystyrene (TCPS) (Figure 3). TCPS was used as a control because serum proteins adsorb well...
on this material, resulting in multiple binding sites for focal cell adhesions. Spread cell morphology on aluminum oxide-coated slides (Figure 4) indicated that proteins adsorbed on this surface facilitate cell binding and that aluminum oxide was not toxic but allowed cell growth. These results correlated well with the known characteristics of alumina, a nearly inert material widely used in hip implants because of its good biocompatibility and excellent corrosion resistance.

Cells proliferated almost as well when cultured in contact with a low concentration of NPs (10 μg/mL) as compared with that of fresh media (Figure 5). This result showed that this particular dose of NPs was innocuous for the cells. These results also correlated well with a previous study in which aluminum oxide NPs (diameter size of 40 nm) at a concentration of 100 μg/mL or lower did not show cytotoxicity. Similarly, Taylor et al. used nanotubes (0.5−40 μm × 40 nm) at a concentration of 10 mg/mL and reported that cytotoxicity and inflammatory response in vitro were improved by coating the nanotubes with aluminum oxide.

At all concentrations, the smaller NPs (NP20) decreased cell proliferation more than the bigger NPs (NP310), in accordance with the previous studies. It should be taken into account that at a specific mass concentration (μg/mL), the surface-to-volume ratio is higher for smaller particles, which implies increased NP−cell interactions and more mobility of the NPs inside of the cell. The cytotoxicity of NPs was shown to be dose-dependent, the threshold concentration depending on overall particle volume instead of absolute particle numbers. The main reason that coated NPs cause cytotoxicity is still not exactly clear. The insolubility of aluminum oxide in neutral aqueous solutions allows a potential toxicity caused by the release of aluminum ions to be discarded. However, NPs can stick onto the cell membrane, damage the cell membrane, or enter into the cells (cytoplasm and/or nucleus) either by endocytosis or by diffusion through the cytoplasm owing to their small size. The interactions between the NPs and the cells may cause apoptosis (programmed cell death), necrosis (traumatic cell death), or just remain in the cell without causing any changes to cell function. NPs may also be responsible in decreasing the amount of cellular antioxidant glutathione (GSH) either by binding to it, inhibiting its synthesis or by depleting the GSH levels.

NPs are known to activate inflammatory cells, whose response could in turn harm surrounding cells such as fibroblasts or osteoblasts. Acute inflammatory response is usually characterized by the release of proinflammatory cytokines (e.g., IL-1β, IL-6, and TNF-α) and an oxidative burst, that is, the release of ROS and nitric oxide. In this study, increased NP−cell interactions and more mobility of the NPs inside of the cell. The cytotoxicity of NPs was shown to be dose-dependent, the threshold concentration depending on overall particle volume instead of absolute particle numbers.

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we correlated the release of ROS to the level of acute inflammatory response of macrophages; evaluating the release of proinflammatory cytokines was beyond the aim of this study.

In good accordance with the cytotoxicity assay, the macrophages released a higher amount of ROS when in contact with smaller NPs (NP20) than with bigger NPs (NP310). This result may be related to the higher amount of NPs that could penetrate into the cells because of their smaller size, in accordance with the previous studies.\textsuperscript{12,26} Regarding the concentration of NPs, the highest concentration (1000 \( \mu \text{g/mL} \)) caused a prominent release of ROS, in contrast with the inexistent release of ROS caused by doses of 10 and 100 \( \mu \text{g/mL} \). This release of ROS in a concentration-dependent manner was in agreement with previous studies.\textsuperscript{23,26,28} The inflammatory effect of the aluminum oxide itself was expected to be low as previously reported by Warashina et al.\textsuperscript{34} after implantation of this ceramic on murine calvarial bone.

This study opens the door to use ALD to coat drug particles with a specific ceramic thickness, which would therefore allow a controlled drug release. A low dose of NPs coated with aluminum oxide was shown to be biocompatible and to induce a negligible amount of ROS.

4. MATERIALS AND METHODS

4.1. Preparation of Samples. Glass slides (Warner Instruments, ref no. 64-0713) and silicon oxide NPs were coated with aluminum oxide (\( \text{Al}_2\text{O}_3 \)) using atomic layer deposition (ALD) (Figure 7). \( \text{Al}_2\text{O}_3 \) coatings were deposited using a SUNALE R-series ALD reactor from Picosun.

TMA (98% pure from ABCR) and deionized water were used as precursors, and nitrogen (99.999% pure) was used as a carrier gas. The reactants were applied in the gas state. During each coating cycle, the reactants were fed for enough time to saturate all active sites. After each pulse of reagent, nitrogen was flushed into the system for purging any unreacted reagent and gaseous byproducts such as methane molecules formed during the reaction. This is an important step to ensure that precursors reacted only on the surface of the growing film. The chemical reactions occurring have been previously described.\textsuperscript{18,35}

All depositions were made at 100 \( ^\circ \text{C} \) and a total pressure of 10 hPa. Depositions on slides were made using 3000 cycles. Before deposition, the substrates were cleaned for 5 min in ethanol in an ultrasonic bath. The deposition on SiO\textsubscript{2} NPs with diameters of 20 nm (Skyspring Nanomaterials, ref no. 6808NM) and 310 nm (\( \sim 2.0 \text{ g/cm}^3 \), MicroSil Microspheres, Bangs Laboratories Inc., ref no. SS02N/10973) was made using 200 cycles. All particles were used as received and distributed as evenly as possible in an aluminum oxide-passivated shallow steel cup, before deposition.

In this study, the word “nanoparticle” (NP) has been used even though commonly NPs are defined as particles having at least one dimension smaller than 100 nm.\textsuperscript{36} However, for simplification, the nomenclature used for the studies with

Figure 6. (a) Normalized luminescence detected when RAW 264.7 was in contact with the NPs (1000 \( \mu \text{g/mL} \) of NP310 and NP20). Luminescence is directly proportional to the release of ROS. NPs at 10 and 100 \( \mu \text{g/mL} \) showed luminescence similar to that of –C and therefore are not shown. (b) Area under the luminescent curve normalized by the negative control. –C stands for negative control (nonactivated cells), and +C stands for positive control. Different letters indicate significant differences between groups (\( p < 0.05 \)). The error bars indicate the standard deviation of triplicate samples.

Figure 7. Scheme of (a) a glass slide and (b) a silicon oxide NP, before and after being coated with \( \text{Al}_2\text{O}_3 \).
particles of nanometric size was NP20 and NP310, where NP stands for nanoparticles and 20 and 310 indicate the diametrical size of the initial NPs in nanometers.

4.2. Characterization of the Coated Slides Using XPS. The chemical composition of the coated slides was analyzed using XPS (Quantum 2000, Al Kα X-ray source, Physical Electronics Inc., USA). A presputtering process was performed to remove the possible contamination on the surfaces. Survey spectra and high-resolution spectra were recorded from areas of size 200 × 200 μm². The relative concentration (atom %) of detected elements was calculated from the relative intensities of peaks, after correction for tabulated sensitivity factors in the software of the instrument. The coated slides were analyzed in triplicate samples.

4.3. Characterization of the Coated NPs Using TEM. TEM was performed using a Jeol JEM 1010 transmission electron microscope. Samples for TEM examination were prepared by soaking a 300 mesh carbon-coated copper grid in a suspension of NPs followed by blotting to remove the excess liquid and air-dried. The NP suspension was obtained by dispersing the NP in Milli-Q water (18 MΩ cm, Q-POD from Millipore).

4.4. Cell Culture Studies. The cytocompatibility was evaluated using human cell lines from two different tissues, hDF and Saos-2. A mouse leukemic macrophage cell line (RAW 264.7) was used as the immune cell model, to evaluate the inflammatory response.

The cells were maintained in cell culture flasks in an incubator under a humidified atmosphere of 5% CO₂ in air at 37 °C. DME/F-12 medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin was used as a culture medium. The culture medium was exchanged every second day. Upon 70%–80% confluence, hDF and Saos-2 were detached and were either used for the experimental study or seeded again in new flasks. Cells were detached with a minimum amount of trypsin 0.25% in EDTA that was inactivated with complete DME/F medium after 10 min. Macrophages were detached by scratching them in a single direction using a cell scraper.

4.4.1. Cytocompatibility Assay. The cytocompatibility assay of the chemical nature of the aluminum oxide coating was evaluated by growing cells of different tissues (hDF and Saos-2) on coated slides. Aluminum oxide-coated slides (θ = 15 mm) were placed in 24-well plates (the slide covering the entire well). hDF and Saos-2 cells were seeded on the coated slides at cell densities of 2000 and 10 000 cells/cm², respectively. As controls, cells were cultured on TCPS with fresh media for the negative control and wells containing no cells were used as blank. After the cells were grown in contact with the NPs for 24 and 72 h, the cell viability was determined using the AlamarBlue assay. Media were removed, wells were washed with PBS (the majority of the NPs were washed out), and 200 μL of 5% AlamarBlue solution diluted in nonsupplemented MEM was added to each well. The well plates were incubated, and the fluorescence was read as previously described.

Triplicates were included in each experiment, and the experiment was repeated three times.

4.4.2. Release of ROS. The release of ROS when a macrophage cell line (RAW 264.7) was in contact with NPs was evaluated because ROS are key signaling molecules induced by acute inflammatory reactions. The release of ROS was monitored through a luminol-amplified chemiluminescence assay. To a white 96-well plate were added 50 μL of NP suspensions (10, 100, 1000 μg/mL) together with 50 μL of nonactivated cells at a cell density of 2 × 10⁵ cells/mL. Both particles and cells were suspended in a protein-free media, that is, 4PBS:1DMEM/100 mM glucose. 100 μL of a luminol solution was dispensed in every well. The luminol solution (500 μM) was prepared by adding 1% luminol (from a stock solution) and 0.2% HRP (1 mg/mL) in a 4PBS:1DMEM/100 mM glucose solution. The luminol stock solution was previously prepared by dissolving 50 mM luminol (3-amino-phenthahydrizide) in 0.2 M NaOH. The controls, without NPs, consisted of nonactivated cells for the negative control and cells activated with 1 μM phorbol-12-myristate-13-acetate (PMA) for the positive control. Media alone were used as blank. The well plate was placed in a microplate reader set at 37 °C. Luminescence was measured every 2 minutes for a total of 60 min, using an integration time of 1000 ms and a settle time of 150 ms. To prevent exposure to light, the experimental procedure was performed in a dark room. Triplicates were included in each experiment, and the experiment was performed using three sets of samples.

The relative amount of ROS generated over the course of the experiment was approximated by calculating the area under the luminescent curve using a numerical integration (eq 1). The luminescence signal of every sample was normalized by the luminescence of the negative control (cell suspension with no NPs).

\[
\text{Area} = \left( t_2 - t_1 \right) \frac{f(t_1) + f(t_2)}{2}
\]

where \( t_1 \) and \( t_2 \) are consecutive measuring times, and \( f(t_1) \) and \( f(t_2) \) are the luminescence values at the respective measuring times.

4.5. Statistics. Statistical analysis was performed in IBM SPSS Statistics 19 (IBM, Chicago, IL, USA) using one-way ANOVA at a significance level of \( \alpha = 0.05 \). Scheffe’s post hoc test was used, and in cases where equal variances could not be confirmed, Tamhane’s post hoc test was used.

5. CONCLUSIONS

ALD has been successfully used to coat aluminum oxide on glass slides and NPs of hundreds of nanometers in size. The coating on NPs was imaged using TEM, and the uniform
aluminum oxide layer was proved using XPS. hDF and Saos-2 were able to grow on aluminum oxide-coated slides and in the presence of coated NPs. Cell viability was only compromised by decreasing the particle size to 20 nm and increasing the concentration of the NPs to 100 μg/mL, which indicated that the NP size and concentration played an important role, whereas aluminum oxide was biocompatible. The release of ROS was shown to be minimal at concentrations equal to or lower than 100 μg/mL, indicating a low acute inflammatory response. These results prove that aluminum oxide coated using the ALD technique does not interfere negatively with the cellular viability of osteoblasts and fibroblasts nor activates the release of ROS by macrophages.

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**Notes**

The authors declare no competing financial interest.

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