Evaluation of the potential of starch-based biodegradable polymers in the activation of human inflammatory cells

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The inflammatory response resulting from the implantation of a medical device may compromise its performance and efficiency leading, in certain cases, to the failure of the implant. Thus, the assessment of the behavior of inflammatory cells *in vitro*, constitutes a key feature in the evaluation of the adverse potential, or not, of new promising biomaterials. The objectives of this study were to determine whether starch-based polymers and composites activated human neutrophils.

Blends of starch with ethylene-vinyl alcohol, with cellulose acetate and polycaprolactone, as well as composites based on all these materials filled with hydroxyapatite have been studied. A lysozyme assay was adapted to examine enzyme secretion from human neutrophils incubated with different starch-based materials. Changes in the free radical and degranulation activity of the neutrophil were also determined by measuring the luminescent response of Pholasin®, a photoprotein that emits light after excitation by reactive oxygen species. The amount of lysozyme secreted by neutrophils incubated with the polymers did not exhibit significant differences between the tested materials. Results were in all cases similar to those obtained for the control (polypropylene) except for one of the starch blends (corn starch with polycaprolactone reinforced with 30% (w/w) of HA).

The chemiluminescence experiments showed that polymers reduce the signal produced by activated neutrophils. Furthermore, for some polymers it was demonstrated that the phenomenon was due to an effect of the surface of the materials in cell adhesion or a simultaneous competition for the photoprotein in solution, which results in the decrease of the intensity of light emitted and detected.

1. Introduction

Following the implantation of any medical device, the wound healing mechanisms are triggered in response to injury and to the presence of a foreign body. The inflammatory response constitutes one of the stages of that complex process aiming to eliminate the cause of injury and any accompanying micro-organisms and to initiate the repair of the surrounding tissues. Biomaterials are not totally inert to the surrounding tissues and thus, an inflammatory reaction is produced by any biomaterial, the severity and duration of which can vary according to the properties of the material [1].

Polymorphonuclear leukocytes (PMNs) are the first cells to arrive at the implant site after surgery. They play a very important role in host defense processes being stimulated by a variety of agents. Their activation may result in several processes such as chemotaxis, phagocytosis, degranulation, and production of O_2^- in a metabolic event known as respiratory burst [2, 3].

Degranulation of neutrophils causes the release of granule contents into the surrounding tissue, which contain human neutrophils peptides, also known as defensins [4]. Defensins perform intracellularly by permeabilizing and killing microorganisms [5] and outside the phagocytic vacuoles by acting as a chemotaxin for monocytes and lymphocytes [6, 7]. Lysosomes contain numerous types of enzymes that are secreted into the tissues during degranulation and frustrated phagocytosis causing severe injury [8]. Neutral proteases such as elastase and collagenase, acid hydrolases and lysozyme are some examples [9–11].

Together with degranulation the microbicidal activity of neutrophils can result from mechanisms dependent on oxygen [12]. The oxygen-dependent mechanisms con-

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sume oxygen as an electron acceptor in reaction initiated by the activation of a multicomponent electron transfer system, the NADPH-oxidase [13]. In this way, toxic unstable superoxide anions are produced which can be dismuted by superoxide dismutase to antimicrobial hydrogen peroxide [14].

A massive and generalized activation of leukocytes may however, impair the host by the excessive release of oxygen radicals and enzymes. This response by leukocytes in the presence of biomaterials can be considered an important measure for biocompatibility for this reason alone. The factors that minimize inflammation will maximize biocompatibility [15]. The multiple responses possible during leukocyte activation and an incomplete understanding of their interactions, lead to the need to measure more than one response to characterize the extent of activation.

Starch-based biodegradable polymers and composites have been proposed for several biomedical applications [16–19]. Biocompatibility studies were already made [20–22] and the aim of this work was to evaluate the potential of these materials in the activation of human inflammatory cells using two complementary techniques.

Neutrophils were isolated from peripheral human blood and challenged, *in vitro*, with different starch-based polymers and composites. The amount of lysozyme released from neutrophils after incubation with the materials was quantified by means of an assay previously adapted for this type of evaluation [23]. The oxidative burst of neutrophils in the presence of the materials was measured by chemiluminescence. Two cell stimulants, formyl-methionyl-leucyl-phenylalanine (fMLP) and phorbol-myristate-acetate (PMA) were used as positive controls for both assays.

2. Materials and methods

2.1. Materials

The materials studied were: (i) a 50/50 (wt %) blend of corn starch and ethylene vinyl alcohol (SEVA-C, Novamont, Italy), (ii) SEVA-C reinforced with 10%, 20%, and 30% (wt) of hydroxyapatite (HA, Plasma Biotal, UK), (iii) a 50/50 (wt %) blend of corn starch and cellulose acetate (SCA, Novamont, Italy), (iv) SCA reinforced with 10%, 20%, and 30% (wt) of hydroxyapatite, (v) a 30/70 (wt %) blend of corn starch and polycaprolactone (SPCL, Novamont, Italy), and (vi) SPCL reinforced with 10%, 20%, and 30% (wt) of hydroxyapatite. In the composites the average size of 90% of the HA particles was found to be below 6.5 μm (laser granulometry analysis).

Poly-L-lactide (Purac biochem bv, The Netherlands), being the gold standard for biodegradables in biomedical applications, was used as a biodegradable control material and borosilicate glass coverslips (BDH, England) for chemiluminescence tests.

All the materials, both the polymers and the composites were processed into circular samples (∅ 1 cm) by injection molding.

2.2. Neutrophil isolation

Neutrophils were isolated from fresh heparinized peripheral human blood collected from healthy volunteers. Blood was mixed with a 6% dextran solution, settled and the supernatant layered onto lymphocyte separation medium and centrifuged at 2400 rpm for 25 min at room temperature. The pellet was washed once with phosphate buffered saline (PBS) solution without calcium and magnesium and the remaining red blood cells were removed by water lysis. The cell suspension was washed twice with PBS without calcium and magnesium at 2400 rpm for 5 min at room temperature. Cells were counted and kept at 4 °C until use.

2.3. Lysozyme assay

The isolated cells were resuspended in PBS with calcium and magnesium at a concentration of 1.5×10^6 cells/ml. Each material was incubated in polypropylene cuvettes with 1 ml of cell suspension for 30 min at 37 °C. A cuvette with cells alone was also used as the negative control. After incubation, the tubes were centrifuged at 2400 rpm for 5 min at 4 °C and 0.5 ml of the supernatants transferred to new tubes to which was added 0.5 ml of *Micrococcus Lysodeikticus* (1.5 mg/ml). These tubes were incubated for 30 min at 37 °C. The lysozyme released by the neutrophils when in contact with the materials breaks down the cell wall of *M. Lysodeikticus* reducing the optical density of the suspension, which can be recorded using a spectrophotometer at a wavelength of 541 nm. In order to quantify lysozyme secretion, a standard curve was prepared with dilutions of neutrophil lysate versus absorbance readings.

2.4. Chemiluminescence

Chemiluminescence is often used to study the neutrophil respiratory burst resulting from biomaterial interactions.

Changes in the free radical and degranulation activity of the cells, were measured by the luminescent response of Pholasin® a photoprotein that emits light after interaction with the reactive oxygen species.

The isolated neutrophils were resuspended in PBS without calcium and magnesium at a concentration of 1×10^6 cells/ml. Two luminometer cuvettes were prepared for each material with 390 μl of HBSS/Hepes buffer, 100 μl of Adjuvant-P™ (Abel® Cell Activation Test, Knight Scientific, UK) and 100 μl of cell suspension. Two tubes with 100 μl of buffer instead of cells were prepared as negative control and another two without any material to work as control in order to verify the activation potential of the cells. All the cuvettes were loaded into the luminometer (Luminometer 1250, LKB Wallace). The first cuvette was then automatically moved to the measuring chamber and 250 μl of Pholasin® (Abel® Antioxidant Test, Knight Scientific, UK) injected, followed by successive cuvettes. The light output from the solution was measured every 250 s for 33 min. At this point, 80 μl of formyl-methionyl-leucyl-phenylalanine (fMLP, 12 $\mu M L^{-1}$) were injected into each cuvette in succession and the light emitted measured for 10 min after which 80 μl of phorbol-myristate-acetate (PMA, 8 $\mu M L^{-1}$) were injected into each tube. Measurements were then taken measuring the light emitted by each cuvette for a total time of 80 min.

Peak luminescent values were determined plotting the amount of light emitted per second (mV) versus time.

2.5. Evaluation of polymers potential to quench light

In order to assess the effect of the materials themselves on the reduction of the signal produced by activated neutrophils an antioxidant assay was performed in the absence of cells but in the presence of free radical donor reagents.

Two replicates of each tube were prepared with 1.335 ml of HBSS/Hepes buffer and 125 µl of Pholasin® and loaded into the luminometer. After 2 min, 40 µl of 3-morpholino-sydnonimine HCl (SIN-1, 2.5 mmolL^{-1}, Abel® Antioxidant Test, Knight Scientific, UK) were injected to each tube. Superoxide and nitric oxide are simultaneously and continually released from the solution of SIN-1. If there are material interactions with the free radicals there will be a delay in the time at which the maximum peak of light is emitted or the magnitude of the peak. Empty cuvettes were the negative control.

2.6. Statistical analysis

All data were averaged and standard deviation is reported as a measure of sample deviation. The effect of the tested materials on the release of lysozyme was compared statistically with Tukey-HDS test [24]. All the materials were compared between themselves and the control. If probability values were less than 0.05 ($p < 0.05$), then the differences observed for the two materials were considered statistically significant.

3. Results and discussion

3.1. Lysozyme

Polymorphonuclear leukocytes comprise one of the immune systems first lines of defence through phagocytosis and destruction of microorganisms. Stimulated phagocytes release lysozomal enzymes and produce a large amount of superoxide anion with the secondary generation of more oxidant species, which result in non-specific damage to surrounding tissues and varying degrees of inflammation.

Several factors influence the phagocytic activity at a biomaterial's interface. It has been shown that the human PMN respiratory burst is influenced by the adhesion to a surface and by the wettability of that surface in the presence or absence of proteins [25–30].

Lysozyme was released by neutrophils after incubation with the degradable materials in study; this was less than 20% of the potential lysed cell maximum for all of the materials (Fig. 1). Furthermore, neutrophils incubated with SEVA-C, all SEVA-C composites, SPCL, SCA reinforced with 10% HA and PLLA did not secrete lysozyme above the negative control (polypropylene test tube). SPCL composites stimulated more enzyme secretion and in fact, the results obtained for SPCL reinforced with 30% of HA are statistically different from those obtained for SEVA-C polymer, all their composites and for PLLA and the control. Also for SPCL with 20% of HA and SEVA-C with 30% of HA, the difference in the amount of lysozyme secreted was significant at the level of 0.05.

The amount of hydroxyapatite in each composite does not correlate with the amount of enzyme secreted in the presence of SEVA-C composites. However, in the case of SPCL, for higher percentages of HA, the quantity of lysozyme released tends to increase. The same type of behavior was observed for SCA composites, although in

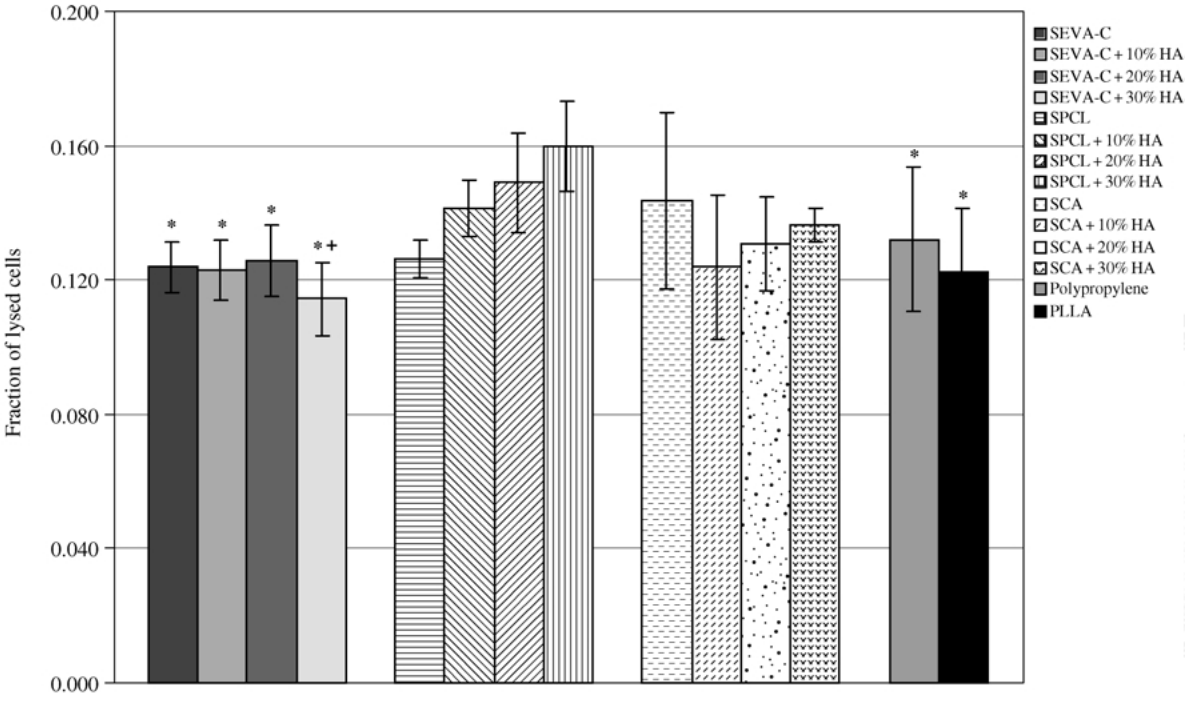

Figure 1 Fraction of *Micrococcus Lysodeikticus* lysed by the lysozyme released by neutrophils incubated with starch-based materials and composites. * Statistically different from SPCL + 30%HA ($p < 0.05$), Tukey–HDS test. $^{+}$ Statistically different from SPCL + 20%HA ($p < 0.05$), Tukey–HDS test.

this case the polymer without any reinforcement induced an even higher percentage of enzyme secretion than the composite with 30% of HA. This seems to indicate that the observed behavior depends on the SCA matrix and when its amount is reduced becomes less intense.

Higher responses have been detected after PMN interaction with hydrophilic surfaces *in vitro* and *in vivo* [28, 31] but in the presence of human serum, neutrophil adhesion and activation is triggered on hydrophobic surfaces *in vitro* [25].

SPCL, SCA and their composites seem to give rise to higher secretion of lysozyme than SEVA-C and its composites. As SPCL and SCA have extreme wettability properties, they are respectively the most hydrophobic and the most hydrophilic of the starch based blends (with a water contact of 70° and 55°, respectively). In fact, the incubation with SCA resulted in higher enzyme secretion, which can be a consequence of a higher cell interaction and activation previously reported for hydrophilic surfaces [28, 31].

One might speculate that SCA composites would induce the highest neutrophil response. The capacity of those materials to uptake water is higher than the polymer without reinforcement due to the interfaces between polymer and HA particles. In fact it has been shown that there is usually a preferential absorption of water and consequent degradation at starch/HA interfaces when its processing is not fully optimized [32]. Thus these particles due to their size, comparing with the matrix, would constitute a preferential site for adhesion, phagocytosis with neutrophils experiencing high levels of degranulation.

PLLA was used as a comparison biodegradable material due to its extensive applications in the biomedical field. It was then possible to observe that the results obtained for starch-based materials were not different from those obtained for the PLLA except in the case of SPCL with 30% of HA. These results indicate that almost all of the starch-based biomaterials (polymers and composites) disclose a behavior at least as good as that of the actual gold standard in the field.

3.2. Chemiluminescence

Neutrophil activation may be either due to a direct effect of the material on the cell membrane or mediated by the adsoption of plasma and matrix proteins [33, 34].

The chemiluminescence assay was used to evaluate the potential of neutrophils to become activated after a direct contact with several biodegradable polymers and composites. The light resulting from the interaction of free radicals and other oxidants, produced by stimulated neutrophils, with the photoprotein Pholasin® was detected and plotted against time. After injection of each cell stimulant, fMLP and PMA, a peak for light emission was observed, as expected, due to an increase in the production of oxygen intermediates. Those two chemicals have two distinct mechanisms of action that explains the differences in the intensity of the peaks of light. The receptor stimulant fMLP works via receptors and acts, in most cases, solely on the NADPH oxidase system of the plasma membrane whereas PMA enters the cells acting directly on protein kinase C, which leads to the activation of the NADPH oxidase both on the plasma membrane and on the secondary granules.

From the results shown in Figs. 2 and 3, it is clear that the maximum response in the chemiluminescence tests was significantly reduced when the cells were exposed to polymers. At the moment of the injection of Pholasin® into the luminometer cuvettes the slight increase in the light detected expected, was not observed for some materials, which seems to show that the phenomena responsible for the reduction of signal is occurring at an early stage of the assay. Furthermore, the response after the injection of the cell stimulants was very low for fMLP and reduced for PMA, about 20% when compared with the positive control.

A mechanism for the down regulation of PMN function was demonstrated in a study by Hansch *et al*. [35], which gave evidence that the dialysis-membrane-associated L-fructose residues participate in a complement-independent neutrophil activation during hemodialysis therapy. This monosaccharide was found to be present in cellulose-based polymers in picomolar concentrations [36].

Moore *et al*. [37] related cellulose acetate degradation with PMN activation *in vitro* and also found that glycerol suppresses reactivity before stimulant addition and after stimulation of neutrophil activation by fMLP or PMA *in vitro* in a dose-dependent manner.

Additionally, it is known that glycerol is used in the manufacturing of starch-based material. This compound may serve to mask a more active inflammatory response to the materials in study since it is the first compound together with low molecular weight chains to leach out from the materials.

It was not possible to observe a tendency in the results either in the case of SEVA-C and composites (Fig. 2) or in the case of SPCL and composites (Fig. 3). It is important to point out the result obtained for PLLA, as it was similar to the negative control.

To verify that the time of the assay was not a limiting factor, the assay was prolonged up to 4 h, but no further changes in the oxidative response of the cells was detected (data not shown).

High levels of Mac-1 expression have been described after contact with surfaces of polymeric materials, which led to increased adhesiveness to the surfaces with a consecutively evoked oxidative burst [38, 39]. However, studies *ex vivo* in a murine model showed that exudate cells respond more to PMA than implant associated cells [26, 40].

To assess whether or not this reduction in the light detected was a consequence of a cell/material interaction or due to possibly free radical interactions with the material, quenching of the light by the material or inhibition of Pholasin®, two types of experiments were carried out.

First, the initial chemiluminescence assay was time-changed. The first light detected in the initial test was almost 4 min after the cuvettes being loaded into the luminometer due to the fact that the luminometer carrousel has to move to the measurement position. Considering this, the number of materials to be tested in each experiment and the number of replicates was reduced, which decreased the analysis time. The results

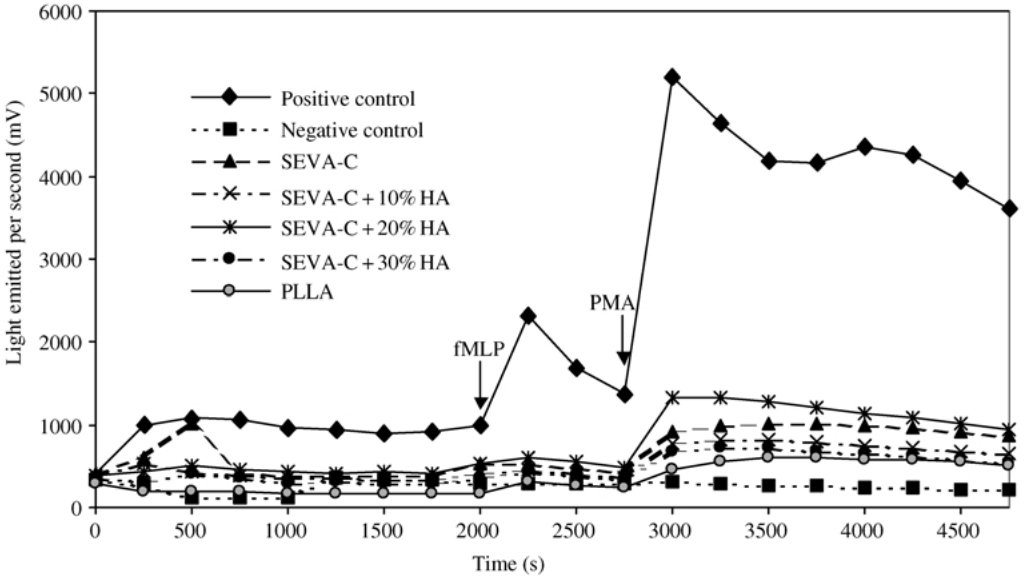

Figure 2 Chemiluminescence measurements of respiratory burst of neutrophils exposed to a starch-based polymer (SEVA-C) and composites and to PLLA. Positive control corresponds to the polypropylene tube (luminometer cuvette) in the same conditions as the tubes with the materials. The negative control corresponds to the luminometer cuvette without cells and buffer instead. Two cell stimulants, fMLP and PMA, were injected to all the cuvettes at different times. Graphs represent mean of $n = 4$ separate experiments, with two replicates of each sample in each experiment.

are presented in Fig. 4; it was possible to observe higher differences between the responses obtained with the different materials and a reduction of signal by polymers. Glass was introduced in this test as a new variable trying to see if different results were obtained for non-polymeric materials. In fact, and as expected, the results were closer to the positive control.

The differences are the greatest when cells were stimulated by PMA especially in the case of glass and SPCL. Cells in contact with those two materials presented a similar response in the beginning of the assay becoming different after fMLP stimulation. SEVA-C, on the contrary, was always the material with the lowest intensity of emitted light.

This same experiment was repeated with 2 min delay in order to verify if the loading and setting time were crucial for the results obtained in the initial chemiluminescence assay. The results are not shown herein but once again changes were detected. For cuvettes with glass and control the same intensity of light was detected but for the tested polymers (SEVA-C and SPCL) the peak of light after PMA stimulation was lower. The same type of kinetics was observed until this point.

Second, nitric oxide and superoxide were released simultaneously and continually from a solution of SIN-1. Those two products react forming peroxynitrite that interacts with Pholasin® in the assay. Light of gradually increasing intensity is detected, reaching a peak after a few minutes (Fig. 5). The results confirm that the materials or any antioxidants capable of scavenging

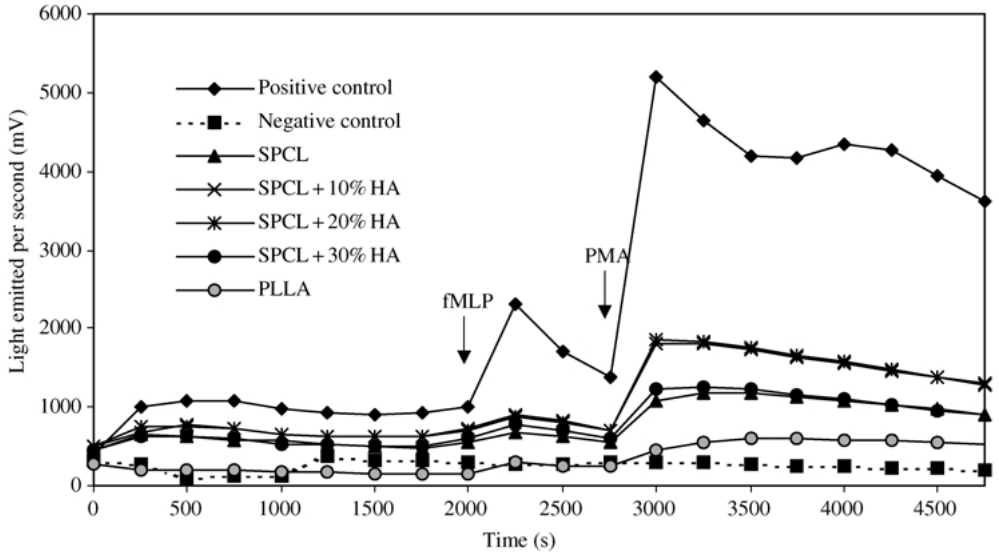

Figure 3 Chemiluminescence measurements of respiratory burst of neutrophils exposed to a starch-based polymer (SPCL) and composites and to PLLA. Positive control corresponds to the polypropylene tube (luminometer cuvette) in the same conditions as the tubes with the materials. The negative control corresponds to the polypropylene tube (luminometer cuvette) without cells. Two cell stimulants, fMLP and PMA, were injected to all the cuvettes at different times. Graphs represent mean of $n = 4$ separate experiments, with two replicates of each sample in each experiment.

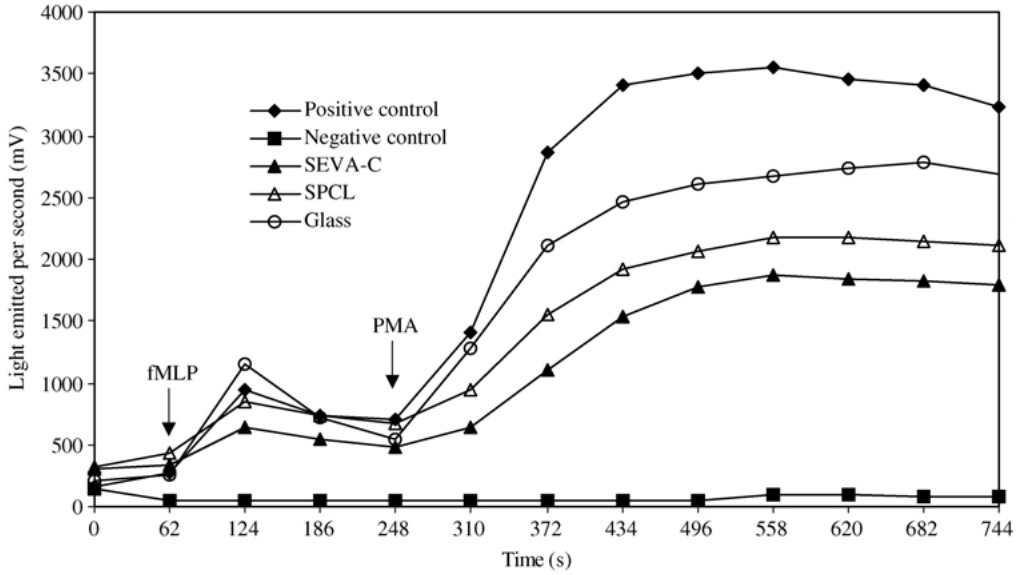

Figure 4 Chemiluminescence measurements of respiratory burst of neutrophils exposed to a two starch-based polymer (SEVA-C and SPCL) and to glass. Positive control corresponds to the polypropylene tube (luminometer cuvette) in the same conditions as the tubes with the materials. The negative control corresponds to the luminometer cuvette without cells and buffer instead. Two cell stimulants, fMLP and PMA, were injected to all the cuvettes at different and earlier times. Graphs represent mean of $n = 3$ separate experiments.

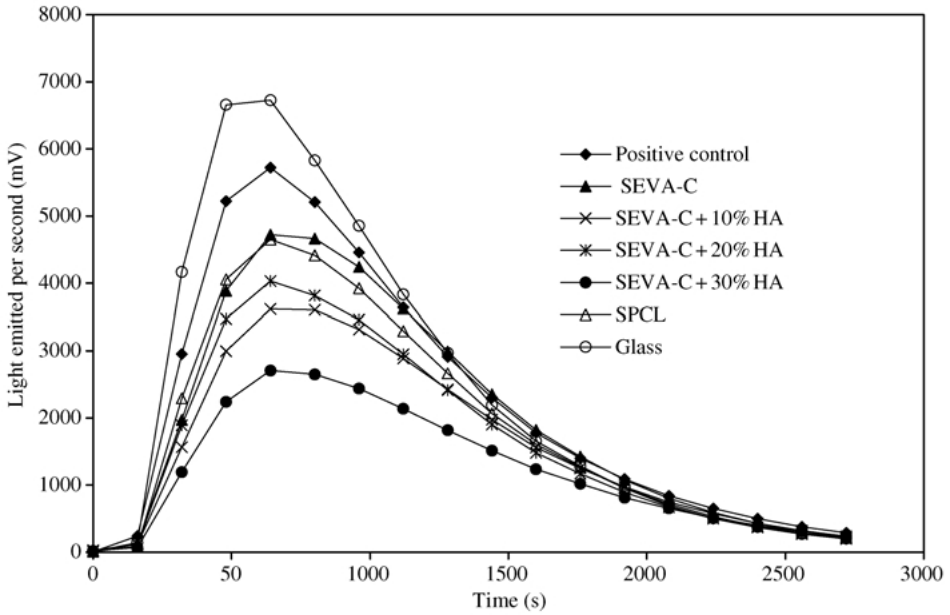

Figure 5 Chemiluminescence measurements of the reaction of peroxinitrite with the photoprotein Pholasin® in the presence of two starch-based polymers (SEVA-C and SPCL), respective composites and glass. Positive control corresponds to the polypropylene tube (luminometer cuvette) in the same conditions as the tubes with the materials. The negative control corresponds to the luminometer cuvette without cells and buffer instead. SIN 1, which promotes the simultaneous production of nitric oxide and superoxide and that react between them originating peroxinitrite, was injected to all the cuvettes. Graph represents one experiment, with two replicates of each sample.

peroxynitrite are competing for Pholasin®, which results in a peak of lower intensity than the control. Furthermore, when comparing the results for SEVA-C and SPCL, as they are similar, the previous differences obtained (Fig. 4) seem to be due to an effect on the adhesion of neutrophils to the surface of those polymers.

There is evidence that indicates that secretion of hydrolytic enzymes and production of oxygen metabolites are directly regulated by a dynamic actin filament system through the association of components of NADPH-oxidase, β2-integrins and actin cytoskeletal structures [25].

In the case of all the materials studied, however, it can be speculated that the reduction of light emitted is the result of the surface properties of the materials on neutrophils adhesion simultaneously with a competition for the photoprotein in solution.

4. Conclusions

Both lysozyme and chemiluminescence assays revealed a low response of the neutrophils when in contact with starch-based polymers and composites.

The hypothesis that the results obtained would be due to an effect on cell adhesion or due to the presence of antioxidant species that would scavenge the reactive

oxygen species, considered so harmful for the tissues, was proved, which allows for considering starch-based materials with weak potential to break out an inflammatory response.

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