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Development and *in vitro* assay of oxidative stress modifying formulations for wound healing promotion

Often presented as metabolism byproducts, reactive oxygen species are linked to detrimental effects such as chronic wound, mutagenesis, cancer and skin ageing. However, recent *in vitro* and *in vivo* observations suggest that ROS, and mainly hydrogen peroxide, interfere with cell signaling acting like second messenger and inducing adaptive responses. This is particularly observed in skin wound healing where cells are exposed to H_2O_2 following injury. In this study, we developed and characterized an innovative formulation producing H_2O_2 at low concentrations, in order to mimic physiological inflammation phase. Then, this pro-oxidative formulation (CAM-GOx) was assayed *in vitro* on keratinocytes cell culture, compared to the blank formulation (CAM) and the anti-oxidative formulation (CAM-CAT) to assess whether oxidative stress was implied or not in cellular responses.

Key words: skin wound healing, hydrogen peroxide, chitosan, alginate, keratinocyte

Reactive oxygen species (ROS) include a variety of free radicals and peroxides, byproducts resulting from aerobic metabolism. These short-lived molecules are generated at low concentration from enzymatic reactions and through leakage from the electron transport chain in the mitochondrion. Exposure to UV radiations or xenobiotics (e.g., drugs, air pollutants, cosmetics, preservatives) generates ROS in excessive quantities resulting in DNA mutation, lipid peroxidation and protein carbonylation [1, 2]. To overcome these damages, cells are equipped with enzymatic systems (e.g. superoxide dismutase, catalase) and radical-scavengers (e.g. glutathione, vitamin C and E, ubiquinone) allowing an efficient detoxification [1]. ROS are involved in pathological disorders such as chronic wound, mutagenesis, cancer, and atherosclerosis but they are also implicated in physiological response during inflammatory phase or wound healing. ROS are potent messenger through modification of transcription factors or signaling molecules, modulating multiple pathways notably the antioxidant response leading to detoxifying enzyme induction [3-5]. Thus, ROS and specifically hydrogen peroxide (H_2O_2), which presents the longest half-life amongst all, are proposed to function as a ubiquitous intracellular messenger at subtoxic concentrations.

Skin wound healing is initiated with the inflammatory phase inducing leukocytes recruitment to the wound site, characterized by a sequential infiltration of neutrophils, monocytes and macrophages [6]. Along with the inflammatory phase, an oxidative stress occurs and H_2O_2 concentrations measured in mice wound fluid increase up to 200 μM two days post injury and was still remarkably elevated five days after injury [7]. This observation is consistent with the kinetic of neutrophils recruitment to the wound site which reach a peak at day two [8]. Leukocyte recruitment to the wound bed primarily aims to clean pathogens, dead cells and necrotic tissue. However, macrophages activity presented a markedly reduced capacity to generate ROS as compared to resident peritoneal macrophages [9] suggesting a more central role in wound healing events through its production of inflammatory cytokines and growth factors [6]. Phagocytic and non-phagocytic cells expressed NADPH oxidases (Nox) in their plasma membrane which generate ROS not as a byproduct but as the main product [1, 10]. Neutrophils are the main ROS producer to the wound site although activated resident cells, e.g. keratinocytes, are able to produce low doses hydrogen peroxide [11].

H_2O_2 has been suggested to induce healing through different *in vivo* observations: i) chronic granulomatous patients,

presenting an impaired NAD(P)H oxidase, are associated with problems in wound healing [12]; ii) monocyte chemotactic protein-1 (MCP-1) deficient mice, unable to attract monocytes to the wound bed, present a delayed healing reestablished after low doses H_2O_2 applications [7, 10]. Moreover, wound healing improvement was suggested to operate through putative hydrogen peroxide induced expression of VEGF [13], TGF-β1 [14, 15], anchorage-independent growth [16] and fibroblast proliferation [17]. Thus, cutaneous wound treatment with topical formulation producing subtoxic doses of H_2O_2 appears as a promising approach to promote healing.

In this study, we developed and characterized a new formulation generating hydrogen peroxide as a function of wound exudate availability. This formulation, as well as the blank formulation and the anti-oxidative formulation were assayed for efficacy on reepithelialization through *in vitro* tests including proliferation, migration, VEGF and TGF-β1 expression.

Materials and methods

Material

Alginate from Macrocystis pyrifera (Sigma-Aldrich, St Quentin Fallavier, France) presented a viscosity in a 2% solution of 237 mPas at 25 °C. Chitosan from shrimp shells (Sigma-Aldrich, St-Quentin-Fallavier, France) presented a viscosity in a 1% in 1% acetic acid solution of 72 mPas at 20 °C. All other reagents were purchased from Sigma Aldrich unless otherwise stated.

Chitosan-coated Alginate microspheres (CAM)

CAM were prepared according to [18, 19] with modifications. A homogeneous dispersion of 44 mg calcium carbonate in 20 mL of 1.5% w/v sodium alginate solution was added to a 60 mL light mineral oil phase containing 500 μL of Span® 80 and 200 μL of Capryol 90™ (Gattefossé, St Priest, France). The obtained w/o emulsion was homogenized 15 min at 400 rpm with an overhead stirrer equipped with a 4 impellers propeller (Yellow Line OST 20 Ika, Germany). Then, an aqueous solution of 0.5% w/v chitosan, 1.0% v/v acetic acid, 1.0% w/v Tween 80, and 0.05 M calcium chloride is added for alginate gelation, emulsion phase breaking and polyelectrolytic deposition of chitosan polymers onto alginate microspheres. CAM were recovered by centrifugation, rinsed with distilled water and suspended to 10 mL final in distilled water.
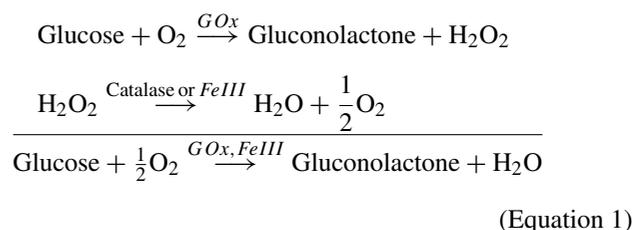
Chitosan-coated alginate microspheres encapsulating enzymes

For the pro-oxidative formulation, glucose oxidase (Type XS from Aspergillus niger, 151,000 U/g) was encapsulated in CAM by dispersing 10 mg of lyophilized enzyme in alginate solution. The CAM-GOx were obtained following the same above described protocol. Thus, the encapsulation efficiency may not be accurately determined by weighting nor protein assay.

For the anti-oxidative formulation, catalase (from bovine liver, 35,100 U/mg proteins) was encapsulated similarly by adding 200 μL of catalase solution to alginate solution. The resulting CAM-CAT, as well as the CAM-GOx, were assayed for enzymatic activity through oximetric methods in order to assess encapsulation.

Oximetric determination of enzyme activity

Encapsulated enzyme activities were determined *in vitro* using an oximetric approach. The activity of glucose oxidase produces hydrogen peroxide with oxygen consumption (Eq.1) while catalase catalyses hydrogen peroxide degradation to oxygen (Eq.1). Thus, oxygen measurement will allow enzymatic activity determination in standardized conditions.

$$\text{Glucose} + O_2 \xrightarrow{GOx} \text{Gluconolactone} + H_2O_2$$

$$H_2O_2 \xrightarrow{\text{Catalase or } FeIII} H_2O + \frac{1}{2}O_2$$
$$\overline{\text{Glucose} + \frac{1}{2}O_2 \xrightarrow{GOx, FeIII} \text{Gluconolactone} + H_2O}$$

(Equation 1)

For GOx activity determination, oxygen measurements were realized with a Clark type electrode (CellOx 325, WTW, Weilheim, Germany) in a closed reactor containing 5.0 mM glucose as substrate, 4.6 μM $FeCl_3$ to catalyze H_2O_2 degradation (Eq.1) and qs 20 mL air saturated distilled water. Basal oxygen concentrations values were recorded during 3 min (Multilab Pilot, WTW, Weilheim, Germany) then 500 μL CAM-GOx suspension were added and oxygen consumption was recorded for 30 min. Oxygen consumption kinetic in *μmol O_2/min/500 μL formulation* was re-expressed as produced hydrogen peroxide, in *μmol H_2O_2/min/500 μL formulation*, then activity was determined as the highest slope of the kinetic curve.

Catalase activity was determined in a closed reactor containing 200 μL of 30% w/v hydrogen peroxide solution in qs 20 mL distilled water depleted in oxygen by gaseous nitrogen bubbling and ultrasonic bath cycling. Oxygen concentration in the reactor was recorded during 3 min, then 500 μL CAM-CAT were added and oxygen production was recorded for 30 min. Activity was determined as the highest slope of oxygen production kinetic and expressed as *μmol O_2/min/500 μL formulation.*

Physicochemical characterization

Particles' zeta potential were determined with a Zetamaster® (Malvern Instruments Orsay, France) using photon correlation spectroscopy (dispersant refractive index = 1.33, detector angle = 90°, wavelength = 670 nm) and electrophoretic measurement (sample dielectric constant = 79, cell field = 28 V.cm^{-1}).

Microspheres' morphology was determined by scanning electron microscopy (SEM) visualization before and after coating with chitosan. Suspended particles were deposited onto carbon adhesive tape and allowed to dry protected from environment. Then, particles were metalized by platinum cathodic sputtering under low vacuum and visualized with a SEM (Hitachi S800 FEG, Centre Technique des Microstructures, UCB Lyon, France).

Cell line and cell culture

CAM, CAM-CAT and CAM-GOx effects on cell cultures were assayed *in vitro*. Keratinocytes of the immortalized and non tumorous cell line HaCaT [20] were used as a model for reepithelialization studies. HaCaT cells were grown as a monolayer on 75 cm^2 tissues culture flasks in DMEM supplemented with 10% v/v FBS and streptomycin-penicillin 100 U/mL each. Cells were incubated in a 5% CO_2 humidified chamber at 37 °C until 90% confluence was reached. Then, cells were unattached with 0.05% trypsin-EDTA and passage was realized. In experiments, cells were previously grown 24 h in serum free defined media (K-SFM) supplemented with 5 ng/mL recombinant epidermal growth factor (EGFr) and 0.05 mg/mL bovine pituitary extract (BPE).

Cell cytotoxicity assay

The viability of HaCaT cells exposed to CAM, CAM-GOx or CAM-CAT was determined by MTT tests. All assays were realized in a 5% CO_2, humidity saturated incubator maintained at 37 °C. Briefly, 5.10^3 HaCaT cells were seeded in 96 wells plate with 200 μL KSFM 24 h before experiment. The following day, media was renewed and cells were treated with increasing concentrations of formulation for 12 h, 18 h or 24 h. Three hours before the end of assay, 100 μg MTT were added to wells enabling viable cells to process MTT to Formazan blue crystals. At the end of incubation period, media was removed and 150 μL of Isopropanol containing 4 mM hydrogen chloride and 0.1% Triton X100 were added allowing cell lysis and formazan solubilization. After 10 min horizontal agitation, absorbances were measured at 595 nm with a multiplate reader. Untreated cells were run as control.

Cell proliferation assay

Proliferative effects of formulations on HaCaT cells were determined through a colorimetric cell proliferation assay based on ELISA determination of incorporated bromodeoxyuridine (BrdU). The assay was run accordingly to manufacturer's recommendations (Cell Proliferation ELISA, BrdU Colorimetric, Roche Diagnostics, France). Briefly, cells were seeded in 96 wells plate with 200 μL KSFM 24 h before experiment at 5,000, 10,000 or 20,000 cells per well. Twenty four hours later, media was renewed and cells treated with increasing concentrations of formulations for 18 h or 48 h. Two hours before the end of incubation time, 10 μM BrdU were added to each wells; BrdU is an analogue of thymidine incorporated in DNA during replication which is detectable with specific antibodies. Incorporated BrdU was determined with the commercial kit resulting in a coloration correlated to the amount of DNA synthesis and hereby to the number of proliferating cells. Absorbance was measured at 450 nm with a multiplate reader.

Cell migration assay

A migration assay was performed in order to determine the effects of formulation on keratinocytes motility. The assay was realized utilizing a commercial kit (Oris Cell Migration Assay, Platypus Technologies, United States) allowing formation of a standardized hole in a confluent layer of cells.

The assay is performed in specific 96 wells plate containing silicon inset designed to permit cell seeding in an annulus shape; the removal of the inset will discover a cell-free disk zone allowing cell from the surrounding annulus to migrate. Keratinocytes from the HaCaT cell line were seeded at 20.10^3 cells per well in KSFM and grown until 80-90% confluence was reached. Subsequently, insets were removed, the wells were rinsed with sterile phosphate buffer saline (PBS) to prevent unattached cells to adhere onto the migration area and cells were treated 24 h with serial dilutions of CAM, CAM-CAT and CAM-GOx in KSFM supplemented with 15 μg/mL fluorouracil (5FU) as antimetabolites preventing cell division. Following 24 h migration, keratinocytes were rinsed with PBS, fixed with 4% paraformaldehyde for 8 min, rinsed again with PBS and stained 4 h with 1 μg/mL acridine orange solution. Cells were rinsed three times with PBS and one time with distilled water before observation with an inverted fluorescent microscope (Olympus IX-50, CCQ, University of Lyon, France). Pictures acquired with a monochrome camera (Scion CFW 1308M, $1,360 \times 1,024$ resolution CCQ, University of Lyon, France) were computed for cell counting (ImageJ, NIH, United States).

Keratinocyte stimulation with CAM's formulations

Keratinocytes from the HaCaT cell line were seeded at 5.10^5 cells in 25 cm^2 culture flasks in 3 mL KSFM for 24 h in a 37 °C, 5% CO_2, humidity saturated incubator. Media were removed from flasks and cells were treated with increasing concentrations of CAM, CAM-CAT or CAM-GOx in KSFM for 24 h. At the end of incubation period, supernatants were recuperated, centrifugated 10 min at 1,500 rpm, sampled in 1.5 mL tubes and frozen at –20 °C until use.

Determination of TGF-β1 expression following CAM, CAM-CAT and CAM-GOx treatments

HaCaT cell expression of TGF-β1 was quantitatively determined by ELISA dosage (Immunoassay kit KAC1688, Invitrogen, France), according to the manufacturer directives. Treated HaCaT cells supernatants were processed to extract TGF-β1 from latent complex and making it detectable. ELISA dosage was performed on samples and standards and absorbance was read at 450 nm with a multiplate reader. The standard curve obtained was plotted with a second degree polynomial equation ($R^2 = 0.998$, Kaleidagraph, Synergy Software) and TGF-β1 concentrations in supernatants were calculated from this equation.

Determination of VEGF expression following CAM, CAM-CAT and CAM-GOx treatments

HaCaT cell expression of VEGF was quantitatively determined by ELISA dosage of the most abundant amongst the five isoforms: VEGF-165 (Immunoassay Kit KHG0112, Invitrogen, France), according to the manufacturer directives. ELISA dosage was performed on samples and standards and absorbance was read at 450 nm with a multiplate reader. A linear regression was applied to the standard curve ($R^2 = 0.987$, Excel, Microsoft) and samples concentrations were calculated from the obtained equation.
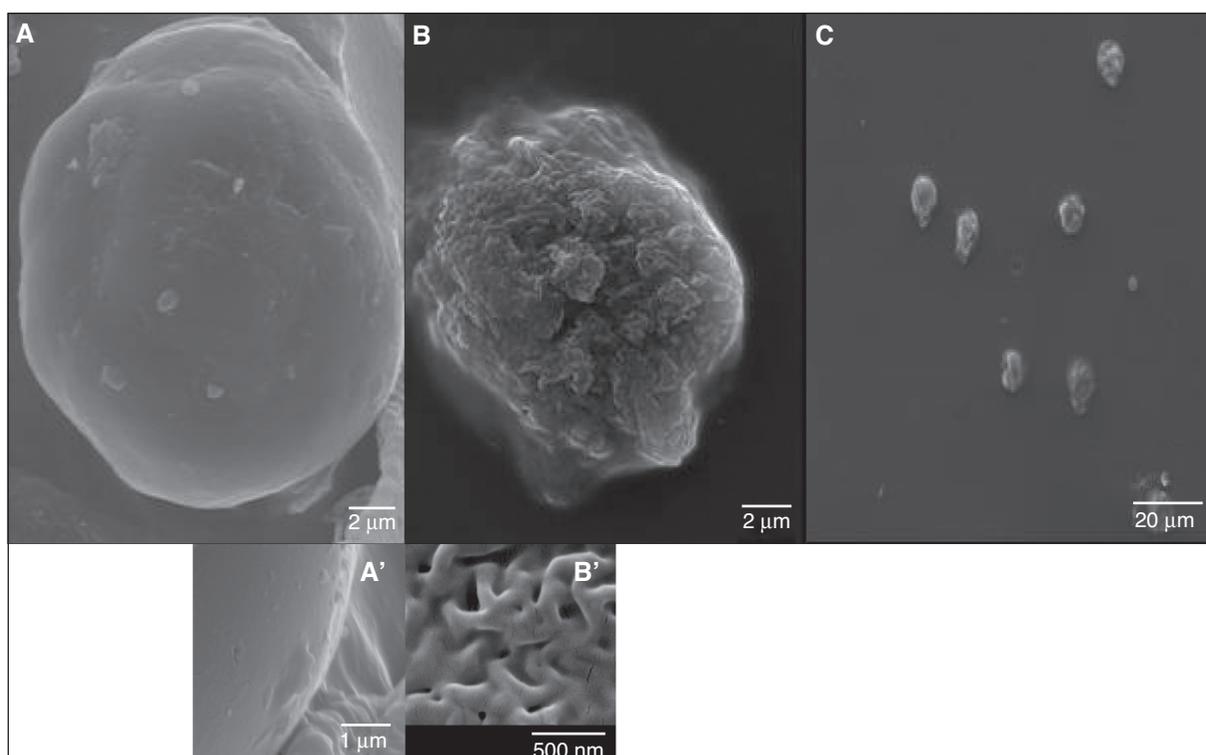
Figure 1. SEM microphotography of alginate core before (A, SEM magnification ×5,000, A', magnification ×12,000) and after (B, SEM magnification ×5,000, B', magnification ×35,000) chitosan coating. Chitosan coated microspheres appear as spherical shape particles with regular size distribution according to limited observations (C, SEM magnification ×490).

Results

Chitosan-coated Alginate microspheres

The particles present an average size of $14.1 \pm 3.1\ \mu m$ estimated on measurement of limited particles (n = 19) on SEM microphotographies. SEM microphotography depicts an effective chitosan coating appearing as a tangled surface as compared to smooth surfaced alginate core *(figure 1)*. The alginate core, retrieved before adding chitosan solution, presents a negative zeta potential ($\zeta = -46.7 \pm 7.9\ mV$) resulting from polyanionic charges of alginate. Following chitosan coating, the CAM particles present a positive zeta potential ($\zeta = 58.6 \pm 1.7\ mV$) confirming again the effective deposition of polycationic chitosan onto microsphere surface.

Oximetric determination of enzyme activity

CAM-GOx exhibited a measurable activity in the reactor assay. The average activity retrieved with eleven distinct formulations is $0.509 \pm 0.077\ \mu mol\ H_2O_2/min/500\ \mu L$ of formulation. This suggests a reproducible production process and an efficient GOx encapsulation. The follow up of one formulation batch kept at $8\,^{\circ}C$ showed no activity diminution against time up to 4 months. However, new batches were prepared once a week for *in vitro* assays and activity was checked before each experiment.
CAM-CAT presented a potent activity, up to $22.25\ \mu mol\ O_2/min/500\ \mu L$ CAM-CAT, during oximetric measurement conducing to oxygen bubble formation in the reactor interfering with the oxygen probe functioning. This rapid degradation of H_2O_2 to O_2 was not due to spontaneous degradation of hydrogen peroxide, which was recorded to $0.031\ \mu mol\ O_2/min$, nor catalysis through polymeric microparticles, which was measured to $0.015\ \mu mol\ O_2/min/500\ \mu L$ CAM. Thus, catalase encapsulation in chitosan coated alginate microspheres was efficient, but due to bubble accumulation on the probe, a quantitative determination of the activity was not accomplished.

Cell cytotoxicity assay

Following 24 hours treatment of HaCaT keratinocyte cells with formulae, only CAM-GOx affected the conversion of MTT to crystal blue Formazan by enzymatic activities as compared to untreated control *(figure 2)*. Interestingly, CAM significantly increased the conversion of MTT by keratinocytes, in a dose dependant manner. As compared to the control value, we found an increase up to 2.3 times with CAM diluted at 1/120 *(figure 2)*. The MTT assay is dependant of the oxidative status applied to cells. Indeed, the CAM-CAT formulation, through its anti-oxidative property, abolishes the effect of CAM and MTT conversion by cells was thus, strictly comparable to untreated control *(figure 2)*. Decreasing treatments time to 18 and 12 hours permitted to recover cell viability with highest dilution of CAM-GOx *(figure 3)*. Dilutions above 1/960 did not affect significantly MTT conversion as compared to untreated control cells.
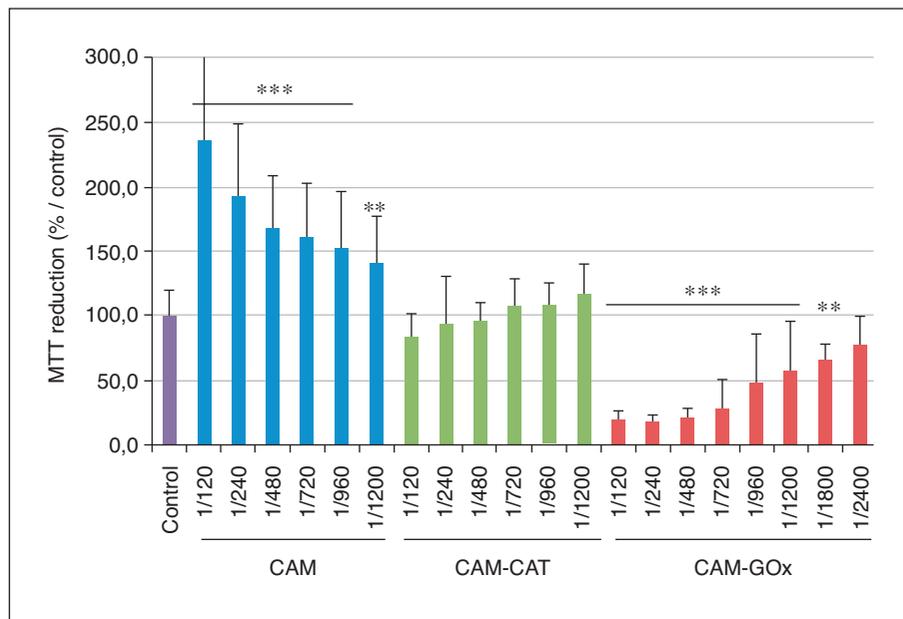
Figure 2. HaCaT cell viability assessed by MTT assay following 24 h treatment. Unloaded chitosan coated alginate microspheres (CAM, blue, mean ± SD of 4 independent assay with 3 batches of formulation and 6 wells per dilution) induces metabolism activation in a dose dependant manner. Interestingly, this effect is abolished with CAM-CAT (Green, mean ± SD of 1 assay with 1 formulation and 6 wells per dilution) while the hydrogen peroxide producing formulation CAM-GOx induces a significant cytotoxicity until 1/1,800 dilution (Red, mean ± SD of 4 independent assay with 3 batches of formulation and 6 wells per dilution). (ANOVA test and Dunnett post hoc, ***$P < 0.0001$, **$P < 0.01$).

Cell proliferation assay

Cell proliferation index was determined by BrdU incorporation assay. Incubation time was lowered to 18 hours in order to minimize cytotoxicity and this impact directly the accuracy of the test. However, BrdU incorporation is a relatively precocious event and detection is sensitive allowing discriminating normally growing cells (control) to cycle-stopped cells (Mitomycin C treated cells). Thereby, as compared to untreated cells, there is an over-all statistically significant increase of BrdU incorporation in cells treated with CAM or CAM-CAT *(figure 4A)* but this observation was not dose dependant, and rising incubation time
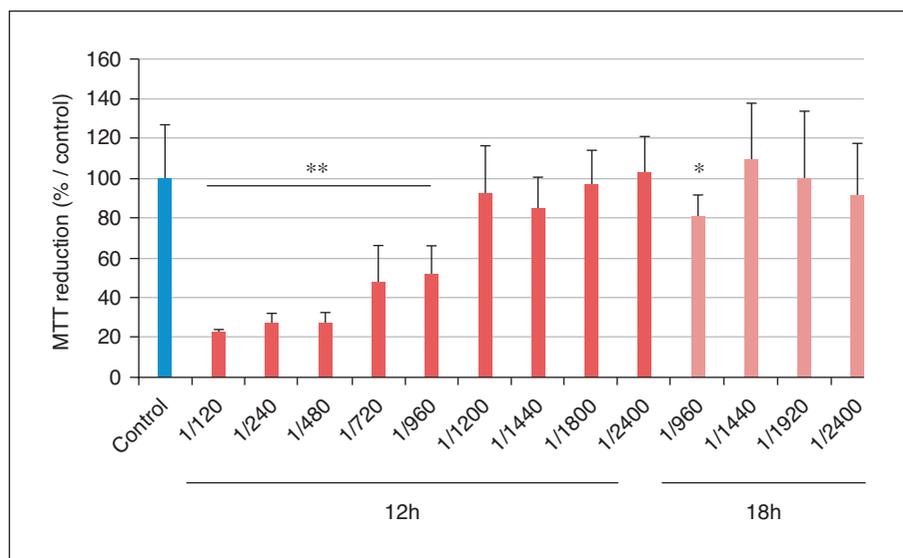

Figure 3. HaCaT cell viability assessed by MTT assay following 12 hours (Dark red, mean ± SD of 2 independent assay and 6 wells per dilution) and 18 hours (Light red, mean ± SD of 2 independent assay and 12 wells per dilution) treatments with CAM-GOx formulation. Decreasing incubation time allow recovery of HaCaT cells viability for dilutions above 1/960 (ANOVA test and Dunnett post hoc, **$P < 0.0001$,*$P < 0.05$).
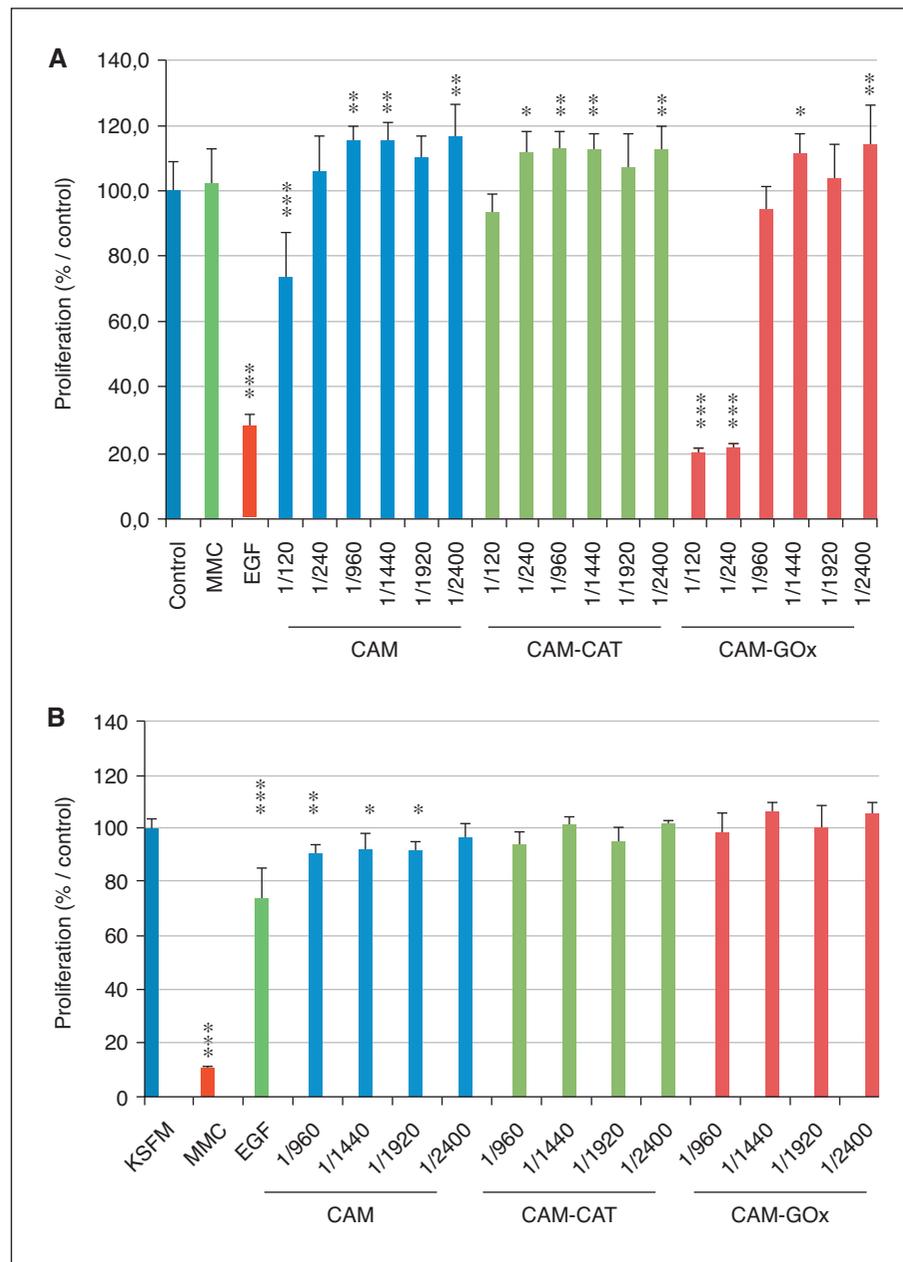
Figure 4. BrdU incorporation in HaCaT cells following 18 hours **(A)** or 42 hours **(B)** treatment with increasing dilution of formulation CAM, CAM-CAT and CAM-GOx. Antimitotic activity of Mitomycin C (MMC, 20 µg/mL) used as a negative control of proliferation, was clearly evidence with this test. Epidermal growth factor (EGF, 10 ng/mL) did not evidence a proliferative effect in our conditions whatever the incubation period (ANOVA test and Dunnett post hoc, $*P < 0.05$, $**P < 0.01$, $***P < 0.0001$).

to 24 hours (data not shown) or 42 hours *(figure 4B)* did not corroborate this observation. Stimulating cells with CAM-GOx did not noticeably influence proliferation, except for cytotoxic dilutions which significantly affect cell viability and thus proliferation *(figure 4A)*. Epidermal growth factor (EGF) is involved in cell growth and proliferation; it is widely used as a proliferation promoter in keratinocytes culture. However, in our culture condition including a 24 hours depletion of EGF before proliferation assay, we did not observe a significant increase of keratinocyte proliferation.

Cell migration assay

Untreated keratinocytes presented an average of 158.5 ± 34.5 cells migrating in free space *(figure 5)*. Treatments with increasing concentration of CAM induced a significant increase in cell migration in free space up to 309.0 ± 43.6 cells with the 1/960 dilution and this was suggesting a dose dependence mechanism. Keratinocytes migration seemed to be controlled by oxidative status as treatments with CAM-CAT was associated with a decrease in migrated cells as compared to the untreated control,
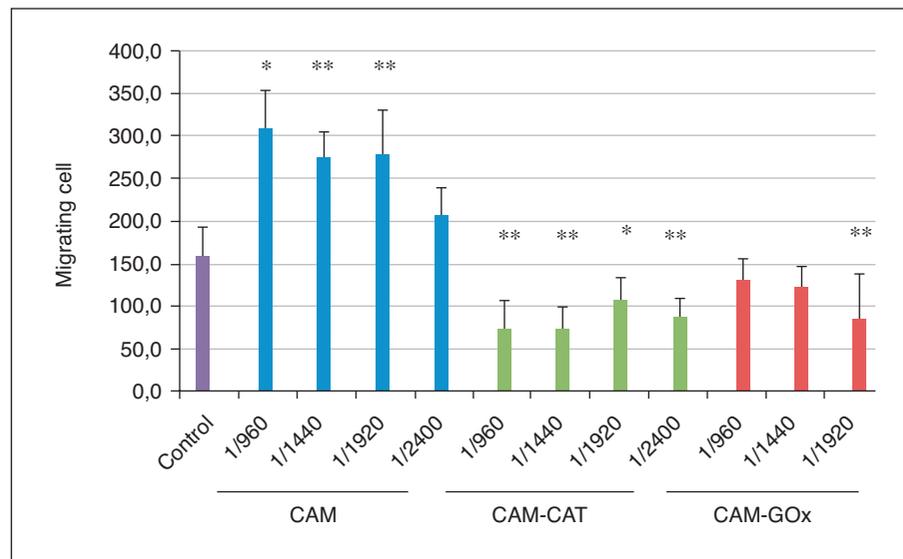
Figure 5. Formulations' effect on HaCaT cells migration. Results are presented as the mean $\pm$ SD of counted cells migrated in standardized open spaces. Treatment with blank CAM significantly increased cells migration. The anti-oxidative property of CAM-CAT abolishes this effect and significantly affected cells migration as compared to untreated control. However, pro-oxidative cells stimulation with CAM-GOx did not conteract CAM-CAT effect as migrated cells were comparable to control.

suggesting that H_2O_2 may play a role in initiating cell migration. CAM-CAT activity abolished CAM induced cells migration and down-regulated basal keratinocyte migration index. However, stimulating cells with CAM-GOx did not potent CAM induced cell migration: excessive oxidative stress abolish CAM induced migration to values similar to untreated cells.

TGF-β1 and VEGF expression following CAM, CAM-CAT and CAM-GOx treatments

TGF-β1 expression in untreated control keratinocytes cells was 103.3 ± 15.6 pg/mL. Treatments with increasing dilutions of tested formulations did not stimulate TGF-β1 expression which was similar among all formulation tested. However, CAM-GOx stimulation with highest concentrations significantly decreased TGF-β1 expression, which may be attributed to cytotoxic effects *(figure 6)*.
Considering VEGF results, concentration in untreated control cells was 631.9 ± 45.9 pg/mL. Treatments with formulations did not evidence a clear effect on VEGF expression in a dose dependant mechanism. Amongst formulations tested and all concentrations, VEGF expressions were similar to control level. Statistically significant increased in VEGF concentrations were found once for each formulation suggesting isolated events as no dose-effects were recorded. CAM induced the highest statistically significant increase of VEGF expression, up to 1.5 times as compared to control; whereas CAM-CAT or CAM-GOx highest increase measured was 1.2 times *(figure 6)*.
On the overall, we did not evidence a direct effect on TGF-β1 nor VEGF expression by keratinocytes following long lasting redox status modification.

Discussion

Microspheres preparation and characterization

Wound healing is initiated with inflammatory phase recruiting leukocytes which in turns create a transient oxidative microenvironment and deliver cytokines activating resident cells to proliferate and migrate in injured tissue. The present study described the preparation of an original H_2O_2 producing formulation auto-adaptable to the wound importance and preliminary assays on *in vitro* keratinocytes cells culture. The formulation developed is a microspheres suspension of alginate cores coated with chitosan (CAM). This system is thought to promote wound healing throughout i) preservation of wound moisture due to swelling properties of alginate and ii) chitosan haemostatic, macrophages activation and cytokine stimulation properties for wound healing promotion [21, 22]. Microspheres were successfully loaded with glucose oxidase or catalase in order to induce a pro-oxidant or an antioxidant activity respectively. Encapsulation effectiveness was assessed by electrochemical oximetric measurement of enzyme activity following microspheres formation. Oximetric measurement method was adapted to our purpose and gave reproducible results comparable to modified Trinder's colorimetric method [23]. This oximetric method was sensible enough to characterize the effect of pH and substrate concentration variations on glucose oxidase activity (data not showed).
CAM-GOx and CAM-CAT presented a measurable activity following preparation including washing centrifugation steps. Thus, encapsulation was effective and the system allows diffusion of substrates and products from enzymatic reaction in CAM particles.
Polyelectrolytic deposition of chitosan polymers onto alginate cores was effective as depicted by zeta potential shift from -46.7 ± 7.9 mV due to polyanionic alginate polymers
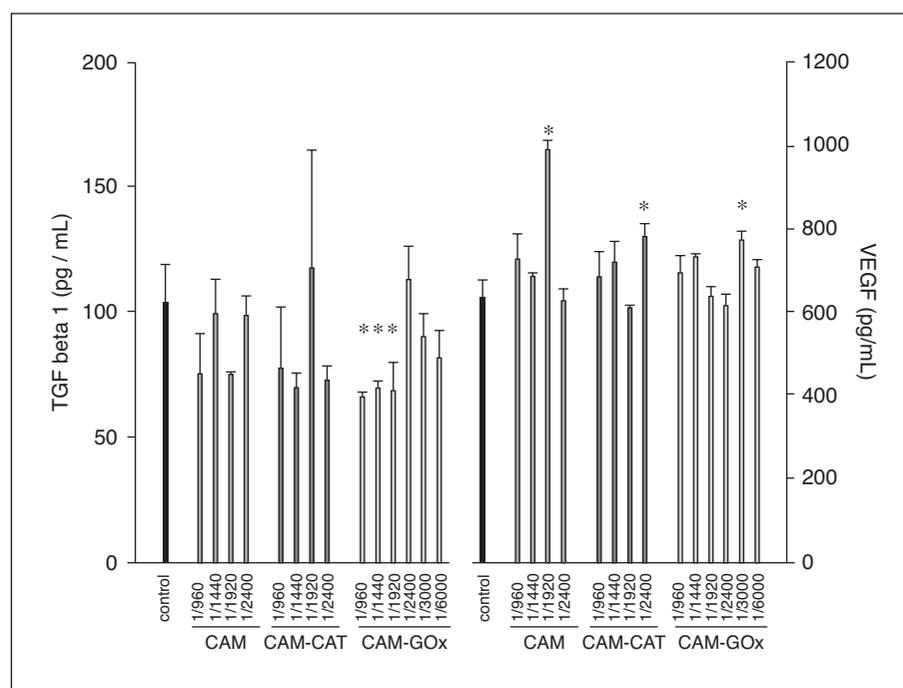
Figure 6. ELISA quantification of TGF-β1 (left) and VEGF (right) expression in supernatant by HaCaT cells stimulated with increasing dilutions of CAM, CAM-CAT and CAM-GOx formulations. Concentrations are the mean ± SD in pg/mL of 2 assays for each dilution. (ANOVA test and Dunnett post hoc, *P < 0.05).

to +58.6 ± 1.7 mV after chitosan deposition. Moreover, SEM microphotographies evidence a round smooth shape for alginate cores and a tangled profile after chitosan deposition.

Chitosan coated alginate microspheres' size was evaluated by calibrated measurements on SEM pictures with ImageJ software. Conjunction of emulsification-internal gelation technique [18, 19, 24, 25] with external gelation and poly-electrolytic deposition of chitosan shorten preparation time and optimized particles' size. Average size estimated at 14.1 ± 3.1 μm was reduced as compared to the average size of 137.5 μm of alginate-chitosan microspheres obtained with a similar method [19]. However, utilizing an emulsification process with solvent and sonication followed by external gelation, Zhu *et al.* obtained an average alginate microspheres size of 4.25 ± 0.49 μm [26].

We successfully developed a methodology for chitosan coated alginate microspheres preparation and loading of glucose oxidase (CAM-GOx) or catalase (CAM-CAT). Those formulations were assayed on keratinocytes culture in order to determine the effect of oxidative stress on epidermal cells.

CAM, CAM-GOx and CAM-CAT effects on keratinocytes

Keratinocytes from the human HaCaT cell line were exposed to different concentrations of formulations and cytotoxic effects were assessed by MTT reduction assay. Interestingly, HaCaT stimulation with CAM induced a higher MTT conversion as compared to untreated controls. Lim *et al.* reported a similar observation with normal human epidermal keratinocytes (NHEK) cells cultured in chitosan porous skin regenerating templates [27]. Similarly, Baruch and Machluf [28] reported a 2.5 times increase of basal human keratinocytes viability when encapsulated in alginate-chitosan microcapsules as compared to non encapsulated cells. However, this observation may not be attributed to enhanced cells proliferation considering the short exposure time but rather to metabolic activation of keratinocytes through alginate and chitosan polymers degradation. Indeed, chitosan polymer may be degraded in chitooligomers by lysozyme, chitinase or chitinase-like enzyme activity, then further hydrolyzed in glucosamine and N-acetylglucosamine monomers which enter metabolic pathway [22, 29, 30]. Activation of metabolism directly influence reduced cofactors turnover and dehydrogenases activity, thus, impacting MTT reduction [31-33]. Interestingly, keratinocyte stimulation with CAM-CAT did not change cell viability as compared to untreated control. Whatever the dilution tested the antioxidant activity of CAM-CAT microspheres abolished or down-regulated the metabolic activation induced by polymers processing observed with the unloaded chitosan-coated alginate microspheres. This supposes that metabolic pathway activation, and thus, MTT reduction increase, is under hydrogen peroxide control. On the other hand, keratinocytes stimulated with CAM-GOx exhibited a statistically significant decreased viability as compared to untreated control. It appears that hydrogen peroxide concentrations achieved were deleterious to keratinocyte cell survival; this was successfully overcome by increasing dilution or decreasing incubation time to 18 hours (no cytotoxic effect for ≥ 1/1,440 dilution) or 12 hours (no cytotoxic effect for ≥ 1/1,200 dilution).

Following stimulation with CAM, HaCaT cells did not exhibit an increased proliferation rate as compared to untreated control. The activated metabolism depicted by MTT reduction assay was not correlated with an increased proliferation profile. However, considering the short time exposure and the absence of positive effect of EGF treatment on cell proliferation, the question arises if HaCaT cells, in our culture conditions, were able to demonstrate a distinct growth following differential stimulations. Nevertheless, the proliferation test was able to discriminate mitomycin C antiproliferation activity and cytotoxic effect of low CAM-GOx dilutions. Increasing proliferation time from 18 to 42 hours did not evidence a proliferative effect of CAM. On the contrary, a limited but statistically significant inhibition of keratinocytes proliferation was found for CAM dilution below 1/2,400. In the mean time, EGF stimulation induced a 30% diminution of cells proliferation as compared to untreated control which is not in line with previous study [34]. Chitosan-coated alginate microspheres are likely to stimulate keratinocyte through: i) chitosan polymers, ii) alginate polymers and iii) calcium ions release from alginate matrix. Chitosan, depending on its deacetylation degree and molecular weight, influence skin cells proliferation and commonly induce a decreased keratinocyte proliferation [35, 36]. Alginate oligosaccharides promote keratinocyte proliferation and have been shown to be as efficient as bovine pituitary extract (BPE) for keratinocyte cultured in EGF containing medium [37]. Alginate cross linked with calcium provides a non negligible source of calcium ions that are known to induce keratinocyte differentiation and down regulation of proliferation [38, 39]. Overall, diminished proliferation observed following HaCaT treatments with CAM is consistent with those reported observations. Adding an antioxidant activity (CAM-CAT) or oxidative activity (CAM-GOx), astonishingly unchanged keratinocyte proliferative activity in our conditions. Except CAM-GOx cytotoxic dilutions, keratinocyte proliferation was slightly increased following 18 hours treatment ($P < 0.05$) and strictly comparable to untreated control following 48 hours treatment.

Keratinocyte migration is a key event in wound reepithelialization, permitting wound closure and skin barrier function regeneration. Reepithelialization implies keratinocytes activation and migration from the wound merges through complex cytokines stimulation and cell-matrix specific interactions with provisional matrix elements. From our results it appears that oxidative status step in HaCaT motility regulation. Treated cells with CAM-CAT formulation exhibited a significantly decreased migration in free space suggesting that the antioxidant activity, due to catalase activity, interferes with HaCaT cells regulation mechanisms and that H_2O_2 is necessary for cell migration. On the over hand, CAM-GOx did not promote keratinocyte migration through H_2O_2 release: the oxidative environment slightly decreases the number of migrated cells as compared to untreated control cells. These observations are in line with recently published work of Nam *et al.* who inhibited HaCaT cells migration with N-acetylcysteine antioxidant but did not promote migration with H_2O_2 treatments [40]. Interestingly, CAM actively promotes keratinocyte migration for dilutions below 1/2,400 up to 2 times as compared to control cells. Low concentrations of calcium are held to be conducive to signal proliferation and migration along

a pathway leading to reepithelialization of the wound site [38]. From this observation it is difficult to assess if calcium alone is sufficient to induce such effect on keratinocyte or if alginate and chitosan polymers induced a specific activity. From our knowledge no specific study has been done on the effects of chitosan or alginate polymers on keratinocytes migration.

Transforming growth factor beta 1 (TGF-β1) is constitutively expressed in keratinocytes and is up-regulated following UV-B irradiation through ROS production or during wound healing [41-44]. TGF-β1 is evidenced to promote scar formation through increased collagen, protein and inflammatory cells accumulation [45]. ELISA dosage of TGF-β1 in keratinocyte culture supernatant did not evidence modification of its expression. TGF-β1 expression was similar to untreated control and only the highest concentrations of CAM-GOx induced a significant decrease of TGF-β1 expression which may be attributed to cytotoxic effects. Thus, none of the tested formulations are expected to induce TGF-β1 mediated scar formation.

Vascular endothelial growth factor (VEGF) activates endothelial cells to promote angiogenesis and is overexpressed by epidermal keratinocytes in cutaneous wound healing [46]. VEGF elevated expression is associated with scarless fetal wounds [45]. VEGF is expressed in normal keratinocytes and is overexpressed in activated, migrating keratinocytes of the wound [47]. Concerning CAM formulation, no effect was pointed out on VEGF expression by HaCaT cells; excluding dilution 1/1920 which was significantly enhanced for VEGF expression as compared to untreated control. Modifying redox status with CAM-CAT or CAM-GOx did not change VEGF protein expression in a dose dependant effect. This is not in line with Brauchle *et al.* study which showed that following treatment with 1 mM H_2O_2, keratinocytes increase VEGF expression [48]. Most of the studies relating oxidants as inducers of VEGF expression (for review see [49]) treat cells with a transient bolus of H_2O_2 while the present study relates a long term stimulation with H_2O_2 produced *in vitro*. The cumulated amount may acts like a concentrated bolus which has been suggested to be antiangiogenic trough the pharmacologic biphasic regulation of VEGF following H_2O_2 stimulation [50, 51].

The present study describes the preparation and characterization of chitosan coated alginate core microspheres as a vehicle for pro-oxidative or anti-oxidative activities regulation of wound environment, through catalase or glucose oxidase activities respectively. The respective formulations presented an effective activity and were tested *in vitro* on keratinocyte HaCaT cell line for potential effects. CAM activated keratinocytes metabolism, as assessed by MTT reduction increase, and this was finely tuned by H_2O_2 concentration as CAM-CAT abolished this effect and CAM-GOx failed to potentiate it. CAM stimulation enhanced cellular motility suggesting a role of oxidant in this cellular response as CAM-CAT significantly affected migration. Thereby, modifying redox environment of cultured keratinocytes with our dedicated formulations did not evidence clues for wound healing endorsement. Redox regulation of cells appears to be finely tuned and CAM-induced metabolic increase may suffice to generate a physiologically active hydrogen peroxide concentration inducing keratinocyte motility and thus, reepithelialization of the skin. ∎

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