Cellular Response of Hydrogel Chitosan-Glycidyl Methacrylate-Xanthan

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We have successfully synthesized a novel hybrid natural-synthetic hydrogel Chitosan-Glycidyl Methacrylate-Xanthan (CTSGMA-X). CTSGMA-X hydrogel was synthesized by two different methods: 1) using a neutral aqueous media during a 4-hour reaction, and 2) using an acidic aqueous media following neutralization. These hydrogels show interesting properties for use in the biomedical field, such as scaffolds for tissue engineering. Encouraging preliminary results of *in-vivo* assays prompted us to investigate the potential inflammatory response of these materials *in vitro*. For this propose, inflammatory tests based upon peroxide and Interleukin-1β assays have been performed. Additionally, keratinocytes cell culture was preformed to assess their viability over several time intervals. This viability has been quantified by the DNA (proliferation) assay. The above analyses show satisfactory results of the CTSGMA-X when compared to pure chitosan.

Introduction.

Synthesis of the hydrogel CTSGMA-X was carried out by two different methods: 1) using a neutral aqueous media during a 4-hour reaction, and 2) using an acidic aqueous media following neutralization [1,2] Both synthesis methods yield reproducible materials with similar properties. The pH is around 6.8, which is a very important parameter when used as implant in the spinal cord of Wistar rats. [3] Preliminary results show that the animals present a very important recovery after 30 days. In-vivo assays results are encouraging to continue parallel studies in order to understand additional secondary reactions in living organisms.

Peroxide and Interleukin-1β assays have been performed to analyze the inflammatory response caused by the material, with satisfactory results. [4]

Additionally, keratinocytes cell cultures have been carried out to asses their viability on several time intervals. This *in-vitro* cellular assays were performed on CTSGMA-X hydrogels, as well as keratinocytes line H413 cell culture over these materials. Their viability has been quantified by DNA assay. [5]

Materials and Methods.

The CTSGMA-X hydrogels were synthesized by two different methods: neutral and acid aqueous media. The stoichiometric molar ratios of CTS:GMA are 1:1, 1:2, 1:3 and 1:4 and labelled as Z11, Z12, Z13 and Z14 respectively; and identified with a C, for the neutralized materials, and H for the neutral reaction.[1,2] A film of Chitosan (0.5%) in acetic acid 0.4M, was used as background.

Polymers Pre treatment.

All materials used for the assays were conditioned prior to use. Material discs of 10 mm diameter were placed in 48-well plates. They were immersed in 1 ml of ethanol (70%) for 15 minutes. The ethanol was removed and washed twice with a sterile phosphate buffer solution (PBS). They were kept overnight in a sterile environment.[2]

Sub-culture of Macrophages.
Mouse macrophages cell line J774A.1 were used in these assays, and were maintained in T75 flasks with a treated surface for ensuring optimal cell attachment and growth. The flask surface was thoroughly scraped, an aliquot of 5 mL of cell suspension was added to a new flask with DMEM (5% fetal Bovine Serum and 1% of antibiotic). The new flask was incubated to 37 ºC with 5% of CO_2.

Peroxide Assay
Macrophages were seeded at a cell density of 2 x 10^5 cells/ml in DMEM onto each polymer disc, and a copper disc was used as a positive control for peroxide release. After one hour the media was removed and replaced with Dichlorofluorescein (DCF). Fluorescence was measured at hourly intervals, by transferring 50 µl aliquots from all wells to a new 96 well plate. The readings were taken over six hours period. The fluorescence plate reader (Fluostar Optima) was adjusted at 485 nm excitation and 520 nm emission. After reading the samples were carefully returned to the original wells.[3]

Interleukin 1β assay (IL-1β)
The assay quantitatively determines the levels of IL-1β in cell culture supernatant. Cells were cultured for 2 and 48 hours on the material samples. Lipopolisaccharide (LPS) was used as a positive control for the Il-1β release at a range of concentrations. The assay was performed and optical density of each well was determined using a microplate reader at 450 nm (measurement) and 540 nm (reference) wavelengths.[3]

Culture of Keratinocytes and DNA Assay for Counting Cells
Keratinocytes line H413 were used and their proliferation was assayed in T75 flasks with surface treatment for ensuring optimal cell attachment and growth, with a DMEM:F12 media supplemented with 50 µl of hydrocortisone per litre of media, and maintained at 37º C in a humidified incubator with 5% CO_2. ***DNA Assay:***cells were seeded at density of 4 x 10^4 cells/ml in DMEM:F12 onto each disc of polymer, additionally glasses were used as a positive control. The times for this assay were 24, 72 and 168 hours, after which, media was removed and samples rinsed with PBS. Each sample was transferred to a separate replicate multiwell plate. Distilled water was added to each new well and samples were freeze-thawed three times. Aliquots of 100 µl of the samples, standards and blank (distilled water) were placed into a 96 well plate and 100 µl of Hoechst Stain was added to each well. The plate was shaken for 10 seconds and fluorescence measurements at 355 nm excitation and 460 nm emission were taken using a fluorescence plate reader (Fluostar Optima).[4]

Results and Discussions.
Figure 1, shows the results obtained for 1 and 6 hours of peroxide assay to evaluate the inflammatory response. In general, macrophages were not activated to release peroxide in response to the hydrogels apart from the response to sample Z11C, although the value was very low compared with that of copper (positive control).

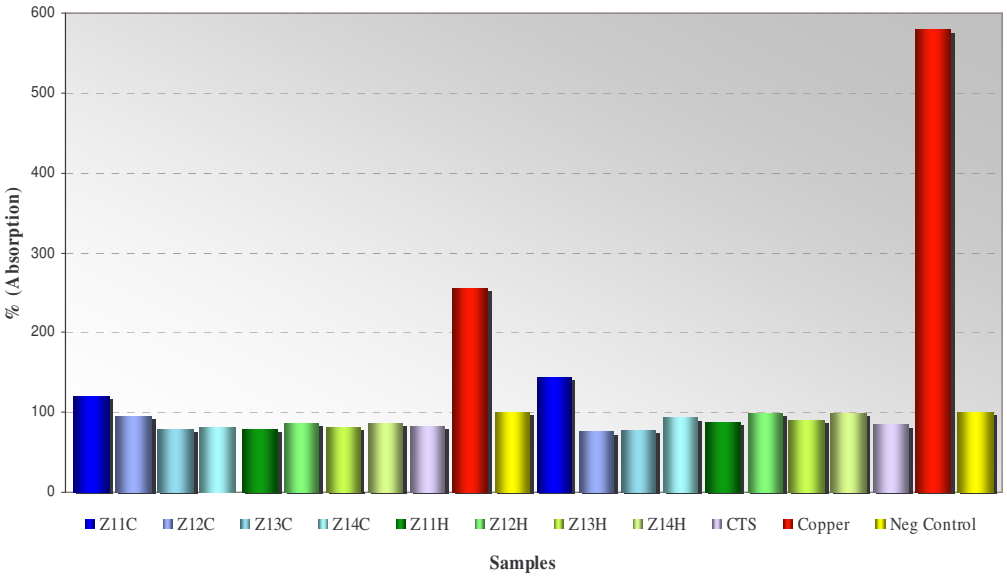

Figure 1. Representative histogram of the obtained values by the peroxide assay, showing to the left results after 1 hour and, to the right, 6 hours.

For the IL-1β assay we have 8 points for the calibration curve from recombinant mouse IL-1β standard. Figure 2 shows the comparative histogram, this graph shows a low concentration of IL-1β after 2 hours of incubation; results showed similar values compared with controls, negative and positive.

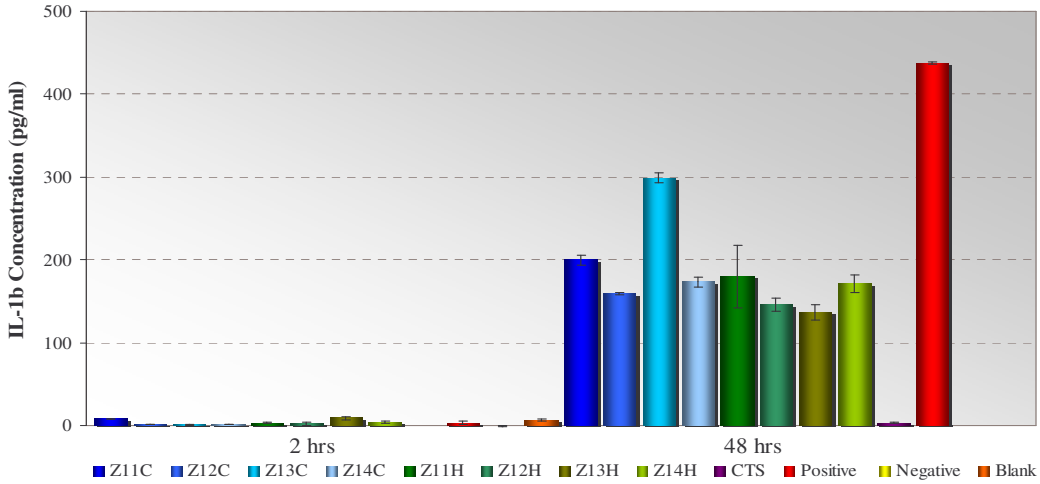

Figure 2. Interleukin-1β production by mouse macrophages J774A.1 after 2 and 48 hours.

For 48 hours an increase was observed in response to Z13C. This sample shows the highest value around 300 pg/ml, the rest of samples have values below 200 pg/ml and Chitosan presents a very low activity, yielding the lowest value compared with the positive control. All samples show a similar behaviour when they were placed in contact with the macrophages, although all of them have high values compared with another authors.

However, the weight of the samples was around 1 mg/ml, whereas other authors used 50 or 500 µg/ml of samples during 18 or 24 hours (table 1).[6-8]

Table 1. Concentrations and values of IL-1β of different polymers based in chitosan compared with the materials.

Material	Dose (μg/ml)	Incubation time (hrs)	IL-1β production (pg/ml)
Oligochitosan[6]	40	18	101.9 ± 46.6
S-DAC70[7]	50	24	28
S-DAC70[7]	500	24	45
Chitosan-DNA[a 8]	-	24	No detected
Z13C	1000	48	298.5 ± 6.1
CTS 0.5%	1000	48	3.2 ± 0.5

[a] With 0.1, 1, 10 and 20 µg of DNA

The figure 3, shows the polymer with macrophages attached in the surface, in addition the picture 3b was observed the material degradation, possible due to resorption of the surface by the macrophage. Analysing the image 3a, the polymer thickness was determined around of 4 µm, meanwhile the spread macrophage around 10 µm.

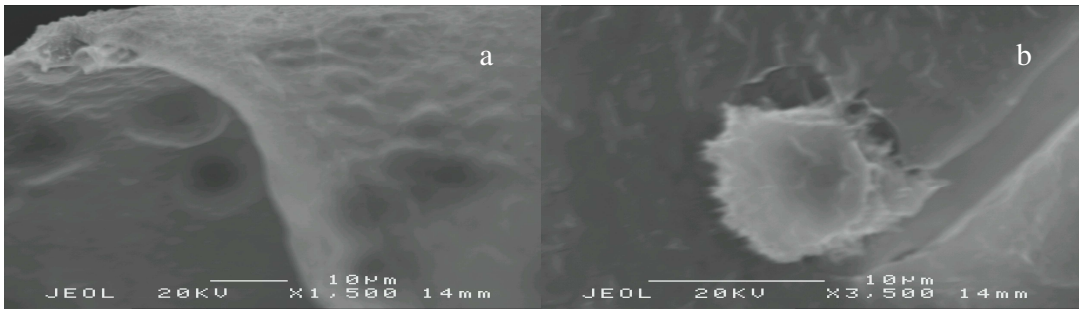

Figure 3. Micrographs showing polymer degradation around macrophage.

These results suggest that macrophages have an active participation in the polymer degradation. This fact is interesting because these materials are proposed as scaffolding for tissue engineering.

The viability of keratinocytes H413 has been quantified by the DNA (proliferation) assay. Figure 7 shows the growth onto biomaterials, the results indicate that some samples (Z14C and Z13H) show a similar behaviour to positive control, increasing the cell number across the time (figure 4). Nevertheless, in general, the hydrogels maintain cell viability better than that of the positive control (glass with a treated surface).

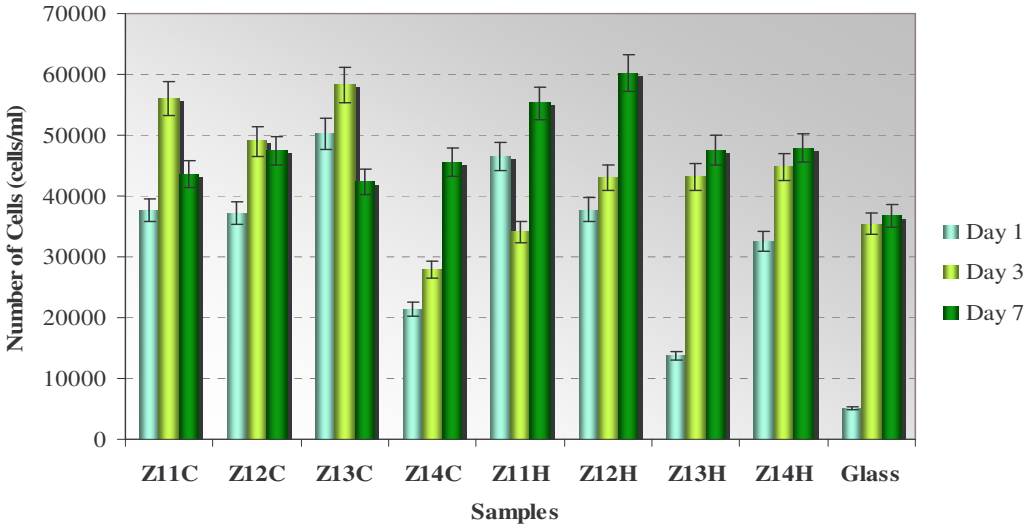

Figure 4. Cell growth of keratinocytes line H413 onto polymers and glass (positive control).

Conclusions.

The results suggest that the materials do not promote the aggressive reaction of macrophages as show in peroxide assay, where the results are below of the negative control for the polymers. For IL-1β the results show a important increase in some of the materials. The DNA assay results indicated that the materials are a potential scaffold for this type of cells (H413), as viability was maintained during 7 days culture with a high number of cells/mL. The results gained from this study an the University of Manchester, UK provide more evidence to propose the use of these materials in the biomedical area. We acknowledge financial support from CONACYT No. 42728

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