Development of cRGDfV-loaded chitosan nanoparticles using factorial design and evaluation of their in vitro cytotoxicity on glioblastoma cell line

Charlene P. Kiill\textsuperscript{1}, Hernane S. Barud\textsuperscript{1}, Leonardo Miziara\textsuperscript{1}, Fabrício Figueirô\textsuperscript{2}, Ana L. R. de Souza\textsuperscript{1}, Ana M. O. Battastini\textsuperscript{2}, Maria P. D. Gremião\textsuperscript{1}

\textsuperscript{1}Univ. Estadual Paulista, UNESP, Araraquara, SP, 14811-060, Brazil; \textsuperscript{2}Universidade Federal do Rio Grande do Sul, UFRGS, Porto Alegre, RS, 90035-003, Brazil char_kiill@yahoo.com.br

ABSTRACT SUMMARY
In this study, it was developed chitosan nanoparticles by ionic crosslinking of chitosan with sodium polyphosphate and characterized in terms of mean particle size (Z-Ave), polydispersity index (PdI) and zeta potential (ZP). Moreover, their in vitro cell viability on glioma cell lines was evaluated.

INTRODUCTION
Glioblastoma multiforme (GBM), the most malignant and invasive glioma, accounts for 15% of all brain tumors. The current treatment for GBM involves surgery, radiation and temozolomide chemotherapy. Moreover, recurrent tumors are chemoresistant. Therefore there is a critical need for new and effective therapies for GBM. Recently, investigations have shown that the snake venom disintegrins, especially those obtained from the venom of the Bothrops alternatus, have been shown to inhibit tumor cell motility and invasiveness. The targeted therapy with the disintegrin is a promising strategy to block integrins signaling pathway. Despite the beneficial effect of disintegrins, this strategy has not been used in clinical trials. The development of cRGDfV-loaded chitosan nanoparticles may promote the passage of drugs/peptides to the central nervous system (CNS) along the olfactory nerve. The aim of this study was the development of CS nanoparticles by ionic crosslinking of CS with sodium polyphosphate (PP), and the characterization in terms of mean particle size (Z-Ave), polydispersity index (PdI) and zeta potential (ZP). The in vitro cell viability of these nanoparticles (NPs) was evaluated on human glioma cell line (U251) and Rattus norvegicus brain glioma (C6).

EXPERIMENTAL METHODS
Experimental factorial design: Two different variables and their influence on the physicochemical properties of CS nanoparticles were evaluated using a 8 full factorial design composed of 3 variables which were set at 2-levels each. The independent variables were the pH, ratio CS:PP (w/w) and acetic acid (M). The established dependent variables were the Z-Ave, PdI and ZP. For each factor, the lower and higher values of the lower and upper levels were represented by a (−1), and a (+1) sign. The data were analyzed using the STATISTICA 10.0 (StaSoft, Inc.) software.

Preparation of NPs: The Nps were prepared following the procedure described by Calvo and coworkers (1997). Briefly, the CS solution (2 mg/mL) was prepared by dissolving in a 0.75% (v/v) acetic acid solution (0.1 and 0.2 M) and leaving it under stirring for 24 h. PP was dissolved in deionised water to a final concentration of 1 mg/mL. Then, the PP solution was added to the CS solution dropwise at different CS:PP ratios (3:0.8 and 3:1) under magnetic stirring at room temperature. cRGDfV disintegrin-loaded nanoparticles were prepared by dissolving the cRGDfV (1 mg/mL) in the PP solution by magnetic stirring at 30 min. The obtained NPs were used for subsequent studies.

Physicochemical characterization Z-Ave, PdI and ZP of RGDfV-loaded and unloaded NPs were determined by using a dynamic light scattering technique (Zetasizer model Nano ZS, Malvern Instruments, UK) with red laser of 633 nm. All samples were diluted with purified water to suitable concentration and analyzed in triplicate.

Cell viability on U251 and C6 cell line glioma: The MTT method provides a quantitative measure of the number of cells with metabolically active mitochondria and is based on the mitochondrial reduction of the tetrazolium bromide salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to a chromophore, the formazan product, whose absorbance can be determined spectrophotometrically. U251 and C6 cell were plated on a 96-well plate at 10\textsuperscript{3} per well, and after reaching semi-confluence, the cultures were treated with 1, 5, 10, 15, or 20 μg/mL of the nanoparticles solution (NP), cRGDfV solution and cRGDfV-loaded nanoparticles for 24 h. After 24 h of treatment, each culture medium containing the compounds was removed, and the cells were washed twice with 100 μL of PBS. After removing the PBS, 100 μL of MTT were added to each of the wells. The cells were incubated for 3 h, and the solution was then removed from the precipitate. A total of 10 μL of DMSO was added to the wells, and the level of absorbance at 570 nm was read using an ELISA plate reader. This absorbance was linearly proportional to the number of live cells with active mitochondria. The cell viability was calculated using Eq. (1): Cell viability (%) = (Abs\textsubscript{in} / Abs\textsubscript{control})*100, Where Abs\textsubscript{in} is the absorbance of cells treated with the different compounds, and Abs\textsubscript{control} is the absorbance of control cells.
RESULTS AND DISCUSSION

The results using the program Statistica show the effect of each parameter (pH, CS:PP and acetic acid concentration) on the average size, PdI and ZP were obtained. With these data it is possible to determine which parameters have a statistically significant influence (ANOVA p < 0.05). The mean particle sizes, PdI and ZP of the cRGDFV-unloaded NPs were reported in Table 1. Particle sizes were mostly between 74 nm to 125 nm (Table 1) and were narrowly distributed. Conjugation with the cRGDFV peptide slightly changed the particle sizes of NPs that were of 150 nm and 250 nm (data not shown in table). Particle size is an important parameter for brain targeting, because particles with diameter less than 200 nm are required for good targeting to the brain. The influence of the independent variables (i.e., pH, ratio CS:PP (w/w) and acetic acid (M)) on the Z-Ave and PdI was checked and demonstrated that the ratio PP (1 and 0.8) have a statistically significant influence (ANOVA p < 0.05) on the Z-Ave and PdI of the NPs.

The ZP showed that the cRGDFV-loaded (data not shown in table) and unloaded NPs have positively charged surfaces with ZP values above 30 mV suggesting good physical stability as described in the literature. The positive charge surface is most likely due to the presence of the CS and cRGDFV in the surface of the NPs. A positive surface is very important for the mucoadhesive properties because the positive charges of the NPs can interact with the sialic groups of mucin, a glycoprotein present in the nasal mucus. This interaction can promote intimate contact and prolong the residence time of the NPs on the nasal mucosa, improving cRGDFV absorption and can allowing delivery of cRGDFV directly from the nasal mucosa to the central nervous system (CNS) along the olfactory nerve.

Table 1. Response values (Z-Ave, PdI and ZP) of the three factors depicted for the cRGDFV-unloaded nanoparticles.

<table>
<thead>
<tr>
<th>pH</th>
<th>Ratio</th>
<th>Acetic Acid (M)</th>
<th>Z-Ave (nm) ± SD</th>
<th>PdI ± SD</th>
<th>ZP ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>(-) 4.4</td>
<td>(+) 0.2</td>
<td>(-) 13 ± 1.3</td>
<td>0.435 ± 0.016</td>
<td>32.2 ± 0.66</td>
<td></td>
</tr>
<tr>
<td>(+) 4.8</td>
<td>(-) 1.0</td>
<td>(+) 112 ± 4.9</td>
<td>0.420 ± 0.022</td>
<td>38.7 ± 0.72</td>
<td></td>
</tr>
<tr>
<td>(+) 4.4</td>
<td>(+) 0.2</td>
<td>(+) 71 ± 1.0</td>
<td>0.283 ± 0.004</td>
<td>31.5 ± 0.28</td>
<td></td>
</tr>
<tr>
<td>(+) 4.8</td>
<td>(+) 0.1</td>
<td>(+) 90 ± 0.25</td>
<td>0.375 ± 0.015</td>
<td>32.6 ± 1.52</td>
<td></td>
</tr>
<tr>
<td>(+) 4.4</td>
<td>(+) 0.1</td>
<td>(+) 114 ± 5.6</td>
<td>0.485 ± 0.107</td>
<td>38.1 ± 1.03</td>
<td></td>
</tr>
<tr>
<td>(+) 4.8</td>
<td>(+) 0.2</td>
<td>(+) 125 ± 3.3</td>
<td>0.469 ± 0.010</td>
<td>37.6 ± 0.45</td>
<td></td>
</tr>
<tr>
<td>(+) 4.4</td>
<td>(+) 0.2</td>
<td>(+) 74 ± 1.6</td>
<td>0.300 ± 0.020</td>
<td>32.2 ± 0.55</td>
<td></td>
</tr>
<tr>
<td>(+) 4.8</td>
<td>(+) 0.2</td>
<td>(+) 82 ± 2.3</td>
<td>0.360 ± 0.051</td>
<td>30.2 ± 0.69</td>
<td></td>
</tr>
</tbody>
</table>

The potential cytotoxicity of cRGDFV solution and encapsulated in nanoparticles against human glioma cell and rattus brain glioma was determined by the MTT assay. As shown in Figure 1, cRGDFV-loaded nanoparticles showed significant cytotoxic effect on U251 and C6 cells. Compared with control group, the viability of U251 and C6 cells treated with cRGDFV in NPs for 24 h was significantly inhibited at the concentration of 20 µg/mL.

CONCLUSION

In general, the results showed that the cRGDFV-loaded nanoparticles could have in vitro cytotoxic effect in glioma, suggesting that the therapy with the disintegrin-loaded NPs is a promising strategy to block integrins signaling pathway and they can be considered a potential candidate for glioma treatment.

REFERENCES


ACKNOWLEDGMENTS

The authors would like to thank FAPESP for financial support and scholarship (Proc. n° 2012/10174-3), CNPq and CAPES for financial support.