Dear Author

Here are the proofs of your article.

- This article has a short turn-around time. We need to receive your corrections within 48 hours. If we do not receive your corrections within 48 hours, we will send you a reminder. Succeeding reminders will be sent every 24 hours until we receive your corrections.

- You can submit your corrections online, via e-mail or by fax.

- For online submission please insert your corrections in the online correction form. Always indicate the line number to which the correction refers.

- You can also insert your corrections in the proof PDF and email the annotated PDF.

- For fax submission, please ensure that your corrections are clearly legible. Use a fine black pen and write the correction in the margin, not too close to the edge of the page.

- Remember to note the journal title, article number, and your name when sending your response via e-mail or fax.

- Check the metadata sheet to make sure that the header information, especially author names and the corresponding affiliations are correctly shown.

- Check the questions that may have arisen during copy editing and insert your answers/corrections.

- Check that the text is complete and that all figures, tables and their legends are included. Also check the accuracy of special characters, equations, and electronic supplementary material if applicable. If necessary refer to the Edited manuscript.

- The publication of inaccurate data such as dosages and units can have serious consequences. Please take particular care that all such details are correct.

- Please do not make changes that involve only matters of style. We have generally introduced forms that follow the journal’s style. Substantial changes in content, e.g., new results, corrected values, title and authorship are not allowed without the approval of the responsible editor. In such a case, please contact the Editorial Office and return his/her consent together with the proof.

- Your article will be published Online First approximately three working days after receipt of your corrected proofs. This is the official first publication citable with the DOI. Further changes are, therefore, not possible.

- The printed version will follow in a forthcoming issue.

Please note

After online publication, subscribers (personal/institutional) to this journal will have access to the complete article via the DOI using the URL:

http://dx.doi.org/10.1208/s12249-014-0115-x

If you would like to know when your article has been published online, take advantage of our free alert service. For registration and further information, go to: http://www.springerlink.com.

Due to the electronic nature of the procedure, the manuscript and the original figures will only be returned to you on special request. When you return your corrections, please inform us, if you would like to have these documents returned.
**Metadata of the article that will be visualized in OnlineFirst**

<table>
<thead>
<tr>
<th></th>
<th>Article Title</th>
<th>Investigation of the Cytotoxic Effects of Titanate Nanotubes on Caco-2 Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Article Sub-Title</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Article Copyright - Year</td>
<td>American Association of Pharmaceutical Scientists 2014</td>
</tr>
<tr>
<td></td>
<td>Journal Name</td>
<td>AAPS PharmSciTech</td>
</tr>
<tr>
<td></td>
<td>Corresponding Author</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Family Name</td>
<td>Bácskay</td>
</tr>
<tr>
<td>6</td>
<td>Particle</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>Given Name</td>
<td>Ildikó</td>
</tr>
<tr>
<td>8</td>
<td>Suffix</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>Organization</td>
<td>University of Debrecen</td>
</tr>
<tr>
<td>10</td>
<td>Division</td>
<td>Department of Pharmaceutical Technology</td>
</tr>
<tr>
<td>11</td>
<td>Address</td>
<td>Nagyerdei krt. 98, Debrecen 4010, Hungary</td>
</tr>
<tr>
<td>12</td>
<td>e-mail</td>
<td><a href="mailto:bacskay.ildiko@pharm.unideb.hu">bacskay.ildiko@pharm.unideb.hu</a></td>
</tr>
<tr>
<td></td>
<td>Author</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>Family Name</td>
<td>Fenyvesi</td>
</tr>
<tr>
<td>14</td>
<td>Particle</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>Given Name</td>
<td>Ferenc</td>
</tr>
<tr>
<td>16</td>
<td>Suffix</td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>Organization</td>
<td>University of Debrecen</td>
</tr>
<tr>
<td>18</td>
<td>Division</td>
<td>Department of Pharmaceutical Technology</td>
</tr>
<tr>
<td>19</td>
<td>Address</td>
<td>Nagyerdei krt. 98, Debrecen 4010, Hungary</td>
</tr>
<tr>
<td>20</td>
<td>e-mail</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Author</td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>Family Name</td>
<td>Kónya</td>
</tr>
<tr>
<td>22</td>
<td>Particle</td>
<td></td>
</tr>
<tr>
<td>23</td>
<td>Given Name</td>
<td>Zoltán</td>
</tr>
<tr>
<td>24</td>
<td>Suffix</td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>Organization</td>
<td>University of Szeged and MTA-SZTE Reaction Kinetics and Surface Chemistry Research Group</td>
</tr>
<tr>
<td>26</td>
<td>Division</td>
<td>Department of Applied and Environmental Chemistry</td>
</tr>
<tr>
<td>27</td>
<td>Address</td>
<td>Rerrich ter 1, Szeged 6720, Hungary</td>
</tr>
<tr>
<td>28</td>
<td>e-mail</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Author</td>
<td></td>
</tr>
<tr>
<td>29</td>
<td>Family Name</td>
<td>Rázga</td>
</tr>
</tbody>
</table>
Titanate nanotubes can be used as drug delivery systems, but limited information is available on their interactions with intestinal cells. In this study, we investigated the cytotoxicity and cellular uptake of titanate nanotubes on Caco-2 monolayers and found that
up to 5 mg/ml concentration, these nanotubes are not cytotoxic and
not able to permeate through the intestinal cell layer. Transmission
electron microscopic experiments showed that titanate nanotubes
are not taken up by cells, only caused a high-density granulation on
the surface of the endoplasmic reticulum. According to these results,
titanate nanotubes are suitable systems for intestinal drug delivery.

Keywords
Caco-2 cells - cytotoxicity - titanate nanotubes

Foot note
information
Investigation of the Cytotoxic Effects of Titanate Nanotubes on Caco-2 Cells

Ferenc Fenyvesi,1 Zoltán Kónya,2 Zsolt Rázga,3 Miklós Vecsernyés,1 Péter Kása Jr.,4 Klára Pintye-Hódi,4 and Ildikó Bácskay1,5

Received 7 November 2013; accepted 13 March 2014

Abstract. Titanate nanotubes can be used as drug delivery systems, but limited information is available on their interactions with intestinal cells. In this study, we investigated the cytotoxicity and cellular uptake of titanate nanotubes on Caco-2 monolayers and found that up to 5 mg/ml concentration, these nanotubes are not cytotoxic and not able to permeate through the intestinal cell layer. Transmission electron microscopic experiments showed that titanate nanotubes are not taken up by cells, only caused a high-density granulation on the surface of the endoplasmic reticulum. According to these results, titanate nanotubes are suitable systems for intestinal drug delivery.

KEY WORDS: Caco-2 cells; cytotoxicity; titanate nanotubes.

INTRODUCTION

Carbon and titanate nanotubes (TiNT) are specific types of nanoparticles (1,2) with the advantage that small particles of active pharmaceutical ingredients (APIs) can be incorporated into the nanotube cavity (3). With nanotube technology, it is possible to prepare stable drug delivery systems, but their safety is a key issue that remains to be resolved. Several publications have reported on the application of carbon nanotubes (4,5), but toxicity studies have not been conclusive (6). Accordingly, the absorption, toxicity and cellular effects of nanotubes should be investigated for a full characterization of their effects. Only limited information is available concerning the cellular effects of TiNT in the gastrointestinal tract, and we have therefore studied their toxicity and absorption through the use of the Caco-2-cell line.

MATERIALS AND METHODS

Preparation of Titanate Nanotubes

TiNTs were synthesized by a simple alkali hydrothermal method involving the alkaline recrystallization of anatase TiO2, as described previously (7,8). The material obtained was characterized by transmission electron microscopy (TEM; Philips CM10, 100 kV), scanning electron microscopy (SEM; Hitachi S4700; Hitachi Scientific Instruments Ltd., Japan) and X-ray diffractometry (XRD; Rigaku miniflex 2000, CuKα). Its specific surface area was determined from nitrogen adsorption measurements performed at 77 K in a Quantachrome Nova 3000e instrument and analysed by the BET method.

Cell Culture and MTT Cell Viability Test

Caco-2 cells were used for permeability and cytotoxicity experiments. Cells were seeded on Transwell® (Corning Costar, USA) filters as reported previously (9). The cellular uptake of TiNT was examined by TEM as described in Fig. 3. To test TiNT cytotoxicity by the MTT method (10), Caco-2 cells were seeded in 96-well plates, and cells were exposed to increasing TiNT concentrations in Hank’s balanced salt solution (HBSS) at 37°C for 120 min. Dye absorbance was measured at 570 nm with a FLUOstar OPTIMA microplate reader (BMG LABTECH, Offenburg, Germany), and the values were corrected for the background absorbance, measured at 690 nm. Cell viability was expressed as a percentage of the untreated control. All reagents were purchased from Sigma-Aldrich (Budapest, Hungary).

Caco-2 Permeability Experiments

In permeability experiments, Caco-2 monolayers were incubated apically with TiNT at 2 mg/ml for 120 min. Then, the permeated amount of Ti was measured with an energy dispersive X-ray fluorescence analyser (Philips MiniPal PW 4025, Philips Analytical, the Netherlands).

Morphology Studies

TEM. The morphology of the synthesized titanate nanotubes was characterized with the aid of a TECNAI G² 20 X-
Twin high-resolution transmission electron microscope operating at an accelerating voltage of 200 kV. Samples for TEM measurements were drop casted onto carbon-coated copper grids from an acetone suspension.

SEM. The surface of the nanotubes was tested with a scanning electron microscope (Hitachi S4700; Hitachi Scientific Instruments Ltd., Japan). A SEM sputter coating unit (Polaron E5100; VG Microtech, UK) was used to charge the surfaces for the SEM measurements.

### RESULTS

The formation of the TiNT was examined by TEM and SEM. Figure 1 shows that the length of the nanotubes was 50–150 nm and their diameter was 6–10 nm. The tubular structure can also be identified. A typical TiNT has four walls and an interlayer of spacing approximately 0.7 nm. The specific surface area of the TiNT is ∼185 m²g⁻¹ due to the specific morphology. The as-synthesized sample (Na-form) exhibited broad peaks of low intensity, which are quite difficult to index, but the profile could be referred to as reflections (around 10°, 25°, 28° and 49°) of sodium trititanate, Na₂Ti₃O₇ (JCPDS no. 31-1329).

Cytotoxic effects could not be detected with the MTT method up to a nanotube concentration of 5 mg/ml (Fig. 2). This is in accordance with the findings that titanium dioxide (TiO₂) nanoparticles at 1 mg/ml did not cause the death of Caco-2 cells (11) and that titania nanotubes up to 1.1 mg/ml are non-cytotoxic on A549 lung epithelial cells (12).

In permeability experiments, no detectable amount of Ti was found in the basolateral side of the monolayers, indicating that intestinal cells are impermeable for TiNT. Monolayers were also treated with 0.5 mg/ml TiNT, fixed with 4% glutaraldehyde and processed for TEM investigations. Nanotubes could not be identified in the Caco-2 cells demonstrating (Fig. 3) that these nanotubes were not taken up by the cells. Nevertheless, high-density granules which had no nanotubular morphology (Fig. 3c, arrows) could be observed on the surface of the endoplasmic reticulum in the treated cells. These granules may be titanium dioxide particles formed from the nanotubes during the incubation or TiO₂ impurities. This is in accordance with a previous report that at ≥10 μg/ml, TiO₂ nanoparticles are able to enter Caco-2 cells and cross a Caco-2 monolayer by transcytosis (11).

![Fig. 1. TEM (a) and SEM (b) images of TiNT](image1)

![Fig. 2. Cytotoxicity of TiNT and TiO₂. Caco-2 cells were treated with nanotubes and titanium dioxide in different concentrations for 120 min, and their viability was determined by MTT tests. Untreated control was considered as 100%, and data are expressed as the percentage of untreated control. Positive control, 2% Triton X-100 solution. There were no significant differences among TiNT- and TiO₂-treated samples (p>0.05), while Triton X-100 exerted complete cell death (p<0.05). Data are means of three independent experiments±SD](image2)
TiO₂ is a safe, widely used excipient in pharmaceutical technology, whereas TiNT is not yet applied. Our study has furnished evidence that TiNT does not cause cellular toxicity in short-term treatment and does not penetrate Caco-2 cells, but does lead to a high-density granulation on the surface of the endoplasmic reticulum. In vitro cytotoxicity assays (i.e., MTT, LDH) can predict irritancy and delayed toxicity of harmful agents (13). Nevertheless, cytotoxicity data alone are not necessarily predictive of in vivo issues (14), but complemented with results of morphology studies, the in vivo toxicity data may be estimated (15). Even if titanate nanotubes are impermeable on intestinal cell layer, they deliver drug particles to intestinal cell surface and can also increase the solubility of active substances. The aqueous solutions are stable for months. That may be the reason that TiNTs provide new possibilities for the formulation of oral drug delivery systems (16).

CONCLUSION

It may be concluded that TiNT is a safe system for intestinal formulations, as they are practically not absorbed from the intestine.

ACKNOWLEDGMENTS

This research was supported by the European Union and the State of Hungary, co-financed by the European Social Fund in the framework of TÁMOP-4.2.2.A-11/1/KONV-2012-0047 and TAMOP 4.2.4. A/2-11-1-2012-0001 “National Excellence Program—Elaborating and operating an inland student and researcher personal support system”.

REFERENCES


AUTHOR QUERIES

AUTHOR PLEASE ANSWER ALL QUERIES.

Q1. Please check the suggested running page title if appropriate. Otherwise, please provide short running title with maximum of 65 characters including spaces.
Q2. Please check affiliations if captured correctly.
Q3. "TiO2 impurities" has been changed to "TiO2 impurities". Please check if appropriate.
Q4. Please check if Ref. 10 was captured correctly.