Design and characterisation of calcium carbonate microspheres for anticancer drug delivery

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Abstract. The development of delivery systems providing prolonged release of antitumor drugs represents one of the challenges in designing and optimization of novel tools for cancer therapy. The employment of spherical inorganic microparticles, in particular, calcium carbonate vaterite microspheres, as microcarriers appears promising because of their porous, matrix structure, biocompatibility, and biodegradability. Here, we summarise the results of the development of the approaches to synthesis of calcium carbonate vaterite microspheres with narrowed size distribution and microencapsulation of low-molecular-weight anticancer drugs, such as doxorubicin hydrochloride into obtained microspheres. Supplementing the reaction mixture with a thickener defines fabrication of homogeneous vaterite microparticles with a spherical shape and an average size of 2 to 3 μm. Synthesised microspheres ensure prolonged release of doxorubicin at physiological pH values and can be used as a delivery system and as a structural component for development of a theranostic platform for tumour treatment and diagnosis.

1. Introduction
Microparticles with a matrix/porous structure represent promising carriers for delivery of antitumor agents with low and high molecular weights e.g., anthracycline antibiotics, alkaloids, and hormones [1, 2]. The encapsulation of drugs in a porous microsphere structure can slow down the dissolution of the drug substances and ensure a longer release from the carrier [3]. This makes it possible to reduce the frequency of anticancer drug administration and, as a result, decrease the risk of adverse reactions and effects [3]. The use of calcium carbonate (CaCO₃) microspheres as matrices is advantageous due to their biocompatibility, biodegradability, susceptibility to pH-dependent erosion [1, 2, 4], and mesoporous internal structure [1], which allows the embedment of different functional components (drugs, bioactive compounds, etc.) [1-3]. These microspheres could be useful for easy and cost-effective production of the delivery systems for cancer treatment [1, 2, 4].

Tuning the conditions of the calcium carbonate microsphere synthesis (temperature, the type and concentration of salt solutions) makes it possible to vary the size of the resultant calcium carbonate microparticles. It also provides an opportunity for fabrication of spherical (vaterite), cubic (calcite), and irregularly shaped (aragonite) microparticles. [4]. Vaterite microspheres can be used as a substrate to prepare polyelectrolyte microcapsules using layer-by-layer adsorption of oppositely charged...
polyelectrolytes [1, 2] and to use them as either a structural component of theranostic agents, or as independent delivery systems [3].

Doxorubicin (DOX) is a strong and therapeutically effective antitumor agent used in the front-line therapy of various cancers, such as ovarian, breast, prostate, lung, and stomach ones; lymphomas; and leukemia. DOX is highly toxic and causes numerous side effects, such as inhibition of hematopoiesis, immunosuppression, and cardiotoxicity [3, 5]. Loading of DOX into microspheres can ensure its controlled and sustained release. DOX is a water-soluble substance; hence, it is challenging to encapsulate it. The creation of a pH gradient when loading DOX in the aqueous medium helps significantly to increase the efficiency of its incorporation into liposomes [5]. However, the method of spontaneous DOX loading [3] or encapsulation at the stage of carrier synthesis, e.g., by the emulsion method [5], is widely used to incorporate DOX into carriers with a matrix structure. Furthermore, the DOX encapsulation approaches that involve the use of the aqueous phase alone are particularly interesting because they require no emulsion stage, organic solvents, or specific equipment for dispersion. The aim of the present study is to determine the conditions for obtaining homogeneous CaCO₃ vaterite microspheres and to establish the approach to effective DOX encapsulation into the microspheres by coprecipitation in aqueous medium. Here, we also present the results of the biopharmaceutical characterisation of the resultant microspheres (e.g., shape, size, surface charge, DOX content, and release at physiological pH).

2. Materials and Methods

2.1. Obtaining of vaterite-type calcium carbonate microspheres
Vaterite-type CaCO₃ microspheres with an average size of 5 to 9 μm were obtained by crystallization (precipitation) from mixed equimolar solutions of Na₂CO₃ (Sigma Aldrich, USA) and CaCl₂ (Sigma Aldrich, USA) as described earlier [6, 7]. Small-sized microspheres were prepared by mixing 7.5 mL of 0.33 M Na₂CO₃ and 7.5 mL of 0.33 M CaCl₂ with equal portions of an 80% (w/v) glycerol solution (Sigma Aldrich, USA). Then the prepared mixture was stirred at 500 rpm and 25°C. The stirring was stopped after 60 min, and the mixture was incubated for 10 min. The resultant microsphere precipitate was washed thrice in ultrapure water. The prepared microspheres were dried at 90°C overnight.

2.2. Encapsulation of doxorubicin into calcium carbonate microspheres
DOX in the form of doxorubicin hydrochloride used in the study was purchased from Sigma Aldrich, USA. The CaCO₃ microspheres containing DOX were obtained by coprecipitation [2]. 1 mL of a DOX solution (containing 0.2 mg, 2 mg, 2.5 mg, 5 mg, 10 mg, or 20 mg of DOX) was added to the mixture consisting of 7 mL of 0.33 M CaCl₂ and 7.5 mL of an 80% glycerol solution. Afterwards, 14.5 mL of the mixture consisting of 7 mL of 0.33 M Na₂CO₃ and 7.5 mL of 80% glycerol was injected into the reaction mixture under stirring. The reaction mixture was stirred at 500 rpm for 60 min at 25°C. Then the stirring was stopped, and the mixture was incubated at room temperature for 10 min. The obtained precipitate was collected and treated as described in Section 2.1.

2.3. Determination of microsphere size and charge
The morphological characteristics and size distribution of the synthesized microspheres were analysed by optical and fluorescence microscopy using an Axio Observer 3 microscope (Carl Zeiss, Germany). The obtained images were processed and analysed in terms of size distribution by means of the Zen software (Carl Zeiss, Germany). The microsphere surface charge was assessed using laser Doppler microelectrophoresis by means of a Zetasizer NanoZS (Malvern, UK).

2.4. Doxorubicin content measurement
~1.27 × 10⁸ microspheres containing DOX were treated with 0.5 M HCl and additionally sonicated to ensure their complete dissolution. The DOX content was measured using spectrophotometry (λmax = 485 nm) by means of a Spark™ 10M multimode microplate reader (Tecan, Switzerland).
2.5 DOX release from microspheres
The DOX release from microspheres was analysed in a 0.05 M phosphate buffer with pH 5.0 or pH7.4 at 37°C. The microspheres containing 0.0045 mg of encapsulated DOX were resuspended in the buffer solution and incubated under the specified conditions while permanently stirring. At specified time points, the supernatant aliquots were collected by centrifugation for quantitative analysis of DOX. Then, the sample volume was replaced with an equivalent fresh portion of the buffer solution preheated to 37°C. The cumulative quantity of the released drug ($Q_{\text{DOX}_{t=0-t}}$) was calculated as following:

$$Q_{\text{DOX}_{t=0-t}} = V_s(C_1 + C_2 + \ldots + C_{t-1}) + V_0C_t,$$

where $V_0$ is the total volume of the release medium, $V_s$ is the sample volume of the supernatant taken for the analysis that was then replaced, and $C_t$ is the DOX concentration at the specified time point. The cumulative release of DOX calculated as the percentage of the initial DOX dose and the release time point were plotted.

3. Results and discussion
The CaCO$_3$ microspheres obtained following the standard approach were spherical in shape and had an average size of 5.6±1.8 μm and a ζ-potential of -18.9±3.27 mV. In order to obtain small-sized carbonate microspheres with an average size in the range from 2 to 3 μm, the reaction mixture was supplemented with a thickener (glycerol solution). Glycerol has been shown to stabilize the vaterite polymorph and promote a decrease in the CaCO$_3$ particle size by increasing the reaction mixture oversaturation and enhancing crystal nucleation due to the larger number of crystallization nuclei [2, 4]. The CaCO$_3$ microspheres obtained with the addition of the glycerol solution exhibited a narrower size distribution and a smaller particle diameter (mean size, 2.7±0.7 μm).

One of the advantages of DOX embedment during the synthesis of calcium carbonate microspheres is the possibility of uniform incorporation of DOX throughout the entire structure of vaterite microspheres due to the adsorption of DOX into the matrix pores during microsphere formation at the maturation stage (Figure 1A). The synthesized drug-containing CaCO$_3$ microspheres had a positive surface charge, a spherical shape, and a size varying from 2.3 to 3.0 μm (Table 1, Figure 1). The presence of an insignificant amount of cubic particles of calcite in the final precipitate can be caused by temperature fluctuations during the synthesis procedure [4]. The quantitative yield of the synthesis product was at least $3.7 \times 10^9$ particles with a drug encapsulation efficiency estimated as at least ~55%. At low loading amounts of DOX (0.2, 2.0, and 2.5 mg), the average drug encapsulation efficiency was estimated as 54.95±2.13%. As the DOX content in the reaction mixture increased (to 5, 10, and 20 mg), its average encapsulation efficiency also enhanced and was determined as 76.41±2.86%. The observed increase in the DOX encapsulation efficiency may be related to reaching a saturation of the DOX solution in the reaction mixture, because the pH of the reaction mixture was alkaline, averaging 9.8-9.9 during the synthesis. Thus, indicates that under the experimental conditions the ionization degree of the DOX hydrochloride salt used for the synthesis decreased and the DOX saturation could be reached due to the drug solubility decrease [5]. The presence of the saturated state of DOX in the reaction mixture was likely to improve the drug encapsulation in the aqueous medium. Earlier, it was shown that incorporation of even 1 mg of DOX into similarly sized calcium carbonate microspheres (2.5 ± 0.2 μm) in a buffered aqueous medium at pH 6.5 by the adsorption technique resulted in a drug encapsulation efficiency not exceeding 29% [3]. Thus, the obtained data demonstrate that the strategy for DOX encapsulation developed here is a promising alternative to the traditionally employed spontaneous drug loading via adsorption.
**Table 1.** Structure and dispersity characteristics of the synthesized doxorubicin-containing calcium carbonate microspheres *

| DOX quantity to be encapsulated, mg | Average microsphere size, µm (n=300) | ζ potential, mV | Microspheres type |
|------------------------------------|--------------------------------------|-----------------|-------------------|
| 1                                  | 2.7±0.8                              | +13.7±0.3       |
| 2                                  | 2.3±0.5                              | +14.9±0.3       |
| 3                                  | 2.9±0.7                              | +14.1±0.2       |
| 4                                  | 2.5±0.6                              | +15.5±0.4       |
| 5                                  | 2.6±0.6                              | +15.7±0.3       |
| 6                                  | 3.0±0.8                              | +18.0±0.4       |

* Mean values for the three independent experiments are presented.

The release of DOX from calcium carbonate microspheres was analysed under the conditions mimicking physiological ones (37°C). The release media with the pH values corresponding to normal (7.4) tissue and local tumour microenvironment or endosome content (5.0) were used for drug release analysis. The results presented in Figure 2 show that the release of DOX from calcium carbonate microspheres could be characterized as prolonged at both pH values. Moreover, the cumulative DOX release reached 75% of the dose fed after 72 h at pH 7.4 and at pH 5.0. The initial release of DOX (during the first 15 min) was found to be of the burst type, which was followed by a release of 20.3±3.7% and 29.9±0.8% of DOX at pH 7.4 and 5.0, respectively. It is important to note that the initial release at pH 7.4 was slightly slower compared to that at pH 5.0. The more intense release of the drug at pH 5.0 at the initial stage could also be explained by the erosion of the microsphere matrix at acidic pH [1].
5

Figure 2. The profiles of doxorubicin release from calcium carbonate vaterite microspheres at pH 5.0 and 7.4 during (A) 72 h and (B) the first 12 h.

4. Conclusion
Thus, we have developed an approach to obtaining small-sized calcium carbonate microspheres and encapsulating DOX into them during the synthesis stage. The obtained microspheres have significantly narrowed size distribution in comparison with microspheres obtained according to the standard precipitation protocol. The developed approach helps to achieve a satisfactory degree of DOX incorporation into microspheres compared to the routinely used spontaneous loading technique due to the specific reaction conditions (pH and viscosity). The obtained microspheres ensure a prolonged drug release when used as microcarriers. The results of the study have shown that the developed microspheres can be used as efficient delivery systems for the antitumour agents of low molecular weight and as a platform for further designing of theranostic agents for cancer treatment and diagnosis.

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