High Loading Efficiency and Controlled Release of Bioactive Immunotherapeutic Proteins Using Vaterite Nanoparticles

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Nanoparticles may limit off-tumor/on-target ubiquitous activation of signaling by protein-based drugs. However, many challenges still exist in the design of a nanoparticle for protein delivery. In this study, conditions to establish vaterite nanoparticles as a pH-sensitive drug delivery system (DDS) for encapsulated protein drugs are comprehensively evaluated. Low coprecipitation pH of vaterite and protein prevents protein denaturation and yields high loading efficiency. Unprotected vaterite recrystallizes in aqueous solutions within 3 h to calcite and releases the loaded protein completely, but surface-modified particles with carboxyl groups containing polymers prove stable for more than 5 months. Notably, modification of vaterite with sulfonated polymers increases the loading of cationic proteins by a multiple. A system is developed for vaterite exposure to (pH) conditions under body-like-flow rates, with the dissolution of vaterite and simultaneous release of active proteins at tumor microenvironmental pH reaching up to 80% and only 20% at physiological pH within 2 h. Importantly, the immunomodulatory protein tumor necrosis factor preserves its native structure and fully retains functional activity in vitro after release from the particles. In conclusion, the studies described here provide a framework for the development of vaterite-based DDS as a carrier for bioactive protein-based therapeutics.

1. Introduction

Nanoparticle (NP)-based drug delivery systems (DDSs) hold great potential for increasing the selectivity and delivery efficiency of therapeutics. In this respect, DDS-delivery has been successfully applied to small molecule drugs, with a liposomal formulation of doxorubicin already approved by the FDA.[1] Progress for therapeutic protein delivery is less advanced, but implementation of DDS-mediated delivery holds considerable appeal as well. Specifically, a DDS can contain the bioactive protein inactive and protected in the core of the nanoparticle while “en route,” with release being triggered at the site where needed under an intrinsic or extrinsic stimulus. This allows for localized activation of agonistic signaling, particularly when combined with functionalization of the surface with targeting ligands to achieve site-directed delivery.[2,3]

Thus, nanomedicine offers a unique chance to overcome some of the challenges associated with current immunotherapeutics, such as on-target/off-tumor effects. Nevertheless, there is a host of critical demands to be met, with the ideal DDS being biocompatible, biodegradable, nontoxic, able to pass physiological barriers, remaining stable during circulation in the blood, and selectively releasing the cargo in the target tissue.[4] Further, the synthesis process should be mild in order to not disrupt the protein structure. Several DDSs for protein delivery are being investigated, including silica,[5] poly(lactic-co-glycolic acid) (PLGA),[6] and liposomes.[7] However, the ideal DDS that can fulfill all these requisites is not yet identified.

A DDS that has the potential to meet most, if not all, of the above-listed criteria is vaterite, a polymorph of calcium carbonate.[8] Vaterite particles are generated using a simple and rapid coprecipitation reaction of calcium carbonate salts within minutes, with modification of the precipitation reaction allowing generation of particles of defined size from micro- to nanoscale.[9] During this simple reaction step, a wide variety of molecules or nanosized payloads can be rapidly loaded by coprecipitation at a high efficiency.[10]

Importantly, vaterite is biocompatible, biodegradable, non-toxic, and has the unique feature of being pH sensitive, with low pH driving the dissolution of particles. This latter feature may be of considerable appeal for the selective release of proteins at acidic conditions, such as the reported pH range of 6–6.5 in the tumor microenvironment.[11–13] CaCO₃ has been explored as a smart DDS for several cancer therapies such as sonodynamic therapy,[14] chemodynamic therapy,[15] and combined thermo–chemotherapy.[16]
For the successful application of vaterite particles in nanomedicine, several issues still need to be resolved. First, the coprecipitation reaction as normally performed for vaterite occurs at a high pH (≈10.3), which can lead to loss of bioactivity of proteins. In this respect, catalase lost 98% of its activity after loading to vaterite microparticles (VMPs) by coprecipitation. Second, spontaneous recrystallization of vaterite into calcite in aqueous solutions leads to immature drug release irrespective of pH. Third, vaterite has a limited ability to load alkaline proteins with high isoelectric point, thereby hampering its universal application.

Here, we comprehensively evaluated conditions for the generation of a pH-sensitive drug delivery system using vaterite particles for encapsulated protein drugs. Specifically, we identified realistic loading conditions to prevent protein denaturation, a strategy to stabilize the unstable vaterite particles up to months and provided a generic and biocompatible approach for increasing the loading efficiency of alkaline proteins. Further, a system to mimic (pH) conditions under body-like-flow rates was developed in order to more accurately predict dissolution and protein release kinetics. The native structure and functional activity of the released protein from vaterite was also evaluated. Taken together, the studies described here provide a framework for use of vaterite-based DDS for bioactive protein-based therapeutics.

2. Results

2.1. Tailoring the Synthesis pH and Size of Vaterite Particles for Loading Bioactive Proteins

Vaterite particles are generated by the precipitation of calcium and carbonate precursors with the most commonly used carbonate precursor being Na₂CO₃ yielding a high reaction pH of 10.3 whereas NaHCO₃ yielding a much lower reaction pH of 5.7. Of note, both reactions yielded similar monodisperse microparticles (for representative pictures, see Figure 1A), with the high coprecipitation pH resulting in higher yield (≈300 mg) and smaller particle size and the low coprecipitation pH resulting in smaller yield (≈100 mg) and larger particle size (Figure 1A).

To delineate the potential impact of coprecipitation pH on loading with bioactive proteins, a small set of proteins of various sizes and diverse charge characteristics were tested at the pH of the respective reaction. Proteins tested comprised lysozyme (Lys) and bovine serum albumin (BSA) as well as the immunotherapeutic proteins tumor necrosis factor (TNF), the EGFR antibody cetuximab (CTX), and the glycan-binding protein galectin-9 (see Table 1 for characteristics). Importantly, exposure of Lys, BSA, TNF, and CTX to the reaction pH of 10.3 encountered during vaterite coprecipitation using Na₂CO₃ induced a significant downward shift in the unfolding temperature.

![Figure 1](https://www.advancedsciencenews.com/figure/image?file=19651_07.png&width=400&height=400)

**Figure 1.** A) Confocal images (on top) depicting vaterite size difference upon synthesis with Na₂CO₃ or NaHCO₃ and FESEM images (below) showing the rough surface of vaterite and the interior porosity of a broken microparticle. Unfolding temperature (UT) of B) Lys and C) TNF, and D) all proteins at different pH values. E) The unfolding temperature of proteins released from vaterite using EDTA (0.2 M) compared to the control recombinant proteins and albumin. F) The average diameter of the vaterite particles plotted as a function of the reaction time for various EG concentrations (inset: confocal image of vaterite nanoparticles, EG 80%, time: 1 h).
Table 1. Characteristics of the various proteins used in this study. Lys (lysozyme), BSA (bovine serum albumin), TNF (tumor necrosis factor alpha), CTX (cetuximab), and Gal9 (galectin-9). pl: isoelectric point.

<table>
<thead>
<tr>
<th>Protein</th>
<th>M_w [kDa]</th>
<th>pl</th>
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<tbody>
<tr>
<td>Lys</td>
<td>14.3</td>
<td>11.35</td>
</tr>
<tr>
<td>BSA</td>
<td>66.5</td>
<td>4.7</td>
</tr>
<tr>
<td>TNF</td>
<td>17.4</td>
<td>5.3</td>
</tr>
<tr>
<td>CTX</td>
<td>150</td>
<td>8.3</td>
</tr>
<tr>
<td>Gal9</td>
<td>35.8</td>
<td>9.3</td>
</tr>
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compared to neutral pH in all proteins (Figure 3B–D; gray vs green lines). In contrast, similar exposure to pH 5.7 did not significantly impact on the unfolding temperature compared to pH 7.4, except for a small downward shift for TNF (Figure 1B–D; orange vs green lines). Thus, the production of vaterite particles at pH 5.7 is better compatible with retaining the native structure of proteins. Indeed, when protein-loaded particles were generated with NaHCO₃ and subsequently dissolved, no difference in protein stability was detected between control recombinant proteins and proteins released from vaterite (Figure 1E).

In addition, the antitumor activity of the glycan-binding protein galectin-9, as previously reported by us for colorectal cancer, was evaluated, with no significant loss in activity after incubation at pH 5.7. Further, we performed an enzyme activity assay for lysozyme, with no loss in enzymatic activity at pH 5 compared to the control sample (revised Figure 1F).

Increasing the stirring speed from an initial 200 rpm with increments up to 1000 rpm significantly decreased the particle region size by ≈27% from 2.9 to 2.1 μm (Figure S1C, Supporting Information). However, in order to attain the nanometer size sought for nanomedicine application, the addition of solvents was used to control the growth of the vaterite crystal. In this respect, previously 80% ethylene glycol (EG) in water solution containing calcium chloride salts was used to reduce the particle size generated using Na₂CO₃ to ≈400 nm.[9] EG is of interest here as it is an additive commonly used for protein stabilization and thus compatible with loading of therapeutic proteins. In line with this data, addition of a dose-range of EG to the reaction mix of NaHCO₃ triggered a clear dose-dependent reduction in size, with 30% EG already reducing particle size to ≈800 nm (Figure 1F). A minimum and consistent vaterite particle size between 350 and 400 nm was detected upon addition of 60%, 70%, or 80% EG during reaction times of 10 to 100 min (Figure S1D, F, Supporting Information; see inset for picture), yielding an ≈12-fold reduction in vaterite particle size.

2.2. Polyelectrolyte (PE) Stabilization of Vaterite Particles

Vaterite particles are intrinsically unstable in aqueous solutions and will easily and irreversibly transform into the thermodynamically stable form calcite.[13] Indeed, in a pH fixed medium (Tris pH 7.4), the micro- and nanovaterite particles rapidly and fully recrystallized within 3 to 5 h to calcite, respectively (Figure 2A). Such a phase transfer in vivo would lead to premature protein release during blood circulation and has to be prevented. To address this issue, vaterite was synthesized in presence of different PEs in order to stabilize vaterite particles in solution (Figure 2B, V-polymer, D1). Vaterite particles synthesized with carboxymethyl cellulose (CMC) proved to be stable for months with no change in morphology, while vaterite particles synthesized with poly(sodium 4-styrenesulfonate) (PSS) did start to degrade from day 12, albeit without detectable recrystallization for months (Figure 2B, V-polymer D60; Figure S1E, Supporting Information). Unfortunately, although the addition of PEs during the coprecipitation reaction stabilized vaterite particles, this also significantly increased particle size (Figure S2A, Supporting Information).

To circumvent this impact on size, vaterite micro- and nanoparticles were instead coated with PEs after synthesis. Coating with only one layer of carboxyl group containing polymers such as CMC, PAA, and PMAA significantly stabilized the vaterite microparticles compared to “naked” vaterite without impact on particle size, with a mixture of vaterite and vaterite debris starting to appear at day 5 (Figure S2C, Supporting Information), without affecting particle size (Figure S2B, Supporting Information). But, vaterite nanoparticles coated with CMC proved to be stable. No trace of calcite could be detected even after 4 months (Figure S2D, Supporting Information). In contrast, polyanions containing sulfate groups such as chondroitin sulfate (CS) and dextran sulfate (DXS) modified could not stabilize vaterite particles (Figure S2C, Supporting Information) while PSS containing sulfonate groups could stabilize them for more than two months. In order to control the release kinetics, BSA-loaded vaterite particles were coated with four double layers of poly(2-methacryloxyethyltrimethylammonium bromide) (PAOET) and CMC, which similarly proved to be stable for months without any change in morphology (Figure 2C, V-BSA-LBL).

2.3. Defining Optimal Conditions for Protein Loading to Vaterite Based on Physicochemical Properties

For loading of proteins in vaterite particles, proteins can either be coprecipitated during vaterite production or loaded postproduction into ready-made particles. Using the coprecipitation method, the proteins TNF and BSA were efficiently loaded with a loading efficiency of 84% and 80%, respectively. However, the loading efficiency of Lys and CTX was only at ≈15% for both proteins (Figure 3A, Cop-MV). Similarly, loading of proteins post vaterite production proved to be very efficient for TNF (70%) and BSA (74%), but again had poor efficiency for CTX (13%) and Lys (11%) (Figure 3A, PL-MV and PL-NV).

It should be highlighted that the loading efficiency did not correlate with protein size (Figure S3A, Supporting Information), but with the isoelectric point (pl). Proteins with high pl have a poor loading efficiency (Figure S3B, Supporting Information; Table 1). Therefore, the pl may be relevant for loading also in view of the fact that the pl of vaterite was experimentally determined to be at pH 7.5 (Figure 3B), with likely charge repulsion of high pl proteins. However, when postloading of Lys and CTX into vaterite microparticles was performed at a pH 9 above the pl of vaterite to overcome charge repulsions, loading efficiency was not improved (Figure S3C, Supporting Information). Thus,
tailoring the buffer pH to alter vaterite charge was not sufficient to increase the loading efficiency.

In an alternate method to change particle charge before postloading of proteins, vaterite particles were modified during coprecipitation with various polyanions. In all cases, addition of polymer generated a negatively charged vaterite particle as measured by zeta potential (Figure 3C). Such a negative charge of the modified vaterite particles was expected to uniformly increase the loading efficiency of alkaline proteins postloading. However, the loading efficiency of Lys in CMC-, dextran sulfate-, CS-, hyaluronic acid (HA)-, or Gelatin B (GelB)-modified vaterite particles was not significantly increased compared to unmodified (pristine) vaterite regardless of their negative charge (Figure 3C). In contrast, vaterite modification with PSS, EGCG, and heparin did strongly increase Lys loading efficiency to 95%, 82%, and 58%, respectively (Figure 3C).

Although PSS and EGCG increased the loading efficiency, these polyanions resulted in insoluble protein precipitates after dissolution of calcium carbonate (Figure 3D). Contrarily, no protein precipitates were observed after the dissolution of heparin-coated vaterite (Figure 4E). Although heparin is biocompatible, it is in clinical use as an anticoagulant and may thus be associated with side-effects when used in NP-based therapeutics. Since heparin belongs to the glycosaminoglycans (GAGs), a group of polymers found in the human body, the other family members hyaluronic acid (HA) and CS were explored for vaterite modification, but these polymers unfortunately failed to increase the loading efficiency of Lys (Figure 3C). Finally, when analyzing alternate sulfate-containing biocompatible polymers, specifically dextran sulfate and dextran with low sulfation degree, the loading efficiency was increased up to 99% (Figure 3C) without any protein precipitates after vaterite dissolution (Figure 3D). DXS-modified vaterite nanoparticles also increased the loading efficiency of other high pI proteins (Figure 3E). Additionally, Lys and CTX preserved their structural conformation after release from DXS-modified vaterite as determined by unfolding temperature, which was identical to that of recombinant control proteins (Figure 3F).

2.4. Dissolution Kinetics of Vaterite in Conditions Mimicking Blood Flow and pH Levels in the Human Body Reveals Preferential Tumor Release

The pH-sensitivity of vaterite particles is a unique feature that can potentially be exploited for selective release of proteins in the acidic tumor microenvironment of pH 6–6.5 versus limited release at neutral pH of 7.4 in the bloodstream. To gain insight into vaterite dissolution kinetics, a controlled system representative of physiological conditions encountered in the human body, particularly in view of (blood) flow conditions, was developed (see schematic in Figure 4A). In brief, vaterite microparticles were loaded in a filter and subsequently subjected to a flowing buffer at a fixed flow rate at relevant pH levels. At a constant flow rate of 0.2 mL min⁻¹ (reminiscent of flow rates in interstitial fluid), the dissolution of vaterite increased in a time-dependent manner within the first 4 min at all tested pH levels (Figure 4B). The lowest dissolution was detected at the physiological pH of 7.4 at 3%, with a significant increase at the tumor microenvironmental pH of 6.5 to 10%. Dissolution further increased to ≈26% at the pH of 4.5 reminiscent of endosomal pH (Figure 4B). Thus, vaterite dissolution kinetics depends strongly on the pH. In addition to pH, the flow rate encountered by vaterite particles in the arteries/arterioles will likely influence the dissolution as well. Indeed, at a flow rate of 1 mL min⁻¹ as would be observed in large blood vessels, the
dissolution of vaterite was significantly higher than at flow rates of 0.5 and 0.2 mL min\(^{-1}\) (Figure 4C). However, our experimental setup exhibit more the situation in the tumor, where the targeting particles are fixed in the tissue and the blood is streaming. In the blood circulation, the relative streaming velocity of blood in respect to the nanoparticles is likely to be close to zero. This will furthermore reduce the dissolution due to the depletion of hydrogen ions in the particle surrounding. Surprisingly there was no significant difference in dissolution kinetics of micro-vaterite (MV) versus nanovaterite (NV) (Figure 4E) pointing to the delivery and temporary concentration of hydrogen ions as limiting factor of dissolution as already presumed by the strong dependence of dissolution kinetics on the flow velocity.

As described above, further modifications of (loaded) vaterite, such as a layer by layer (LbL) coating can be used to increase vaterite particle stability and may also be used as platform for surface modification for, e.g., development of active tumor targeting. Such an LbL coating of vaterite with double layers of PAOET and CMC (PAOET/CMC\(_2\)) did not impact on the kinetics of vaterite dissolution (Figure 4D, MV-RT-LbL). Further, compared to dissolution at room temperature, the dissolution at the body temperature of 37 °C was decreased by 10% at pH 7.4 and 20% at pH 6.5 (Figure 4D, MV-37 °C), with no significant difference in dissolution kinetics of MV versus NV (Figure 4E).

The release of BSA from pristine vaterite particles reached up to ≈80% at pH 6.5 and 24% at pH 7.4 within 2 h in good correlation with the vaterite dissolution (Figure 5A, continuous lines). However, release of BSA from LbL-coated vaterite was markedly lower, with ≈40% release at pH 6.5 and 15% release at pH 7.4 (Figure 5A, dashed lines) and did not correlate with the vaterite dissolution (Figure 5B, black dashed lines vs continuous red lines, respectively). The latter was almost not influenced by the LbL capsule.

2.5. Bioactivity of the Immunotherapeutic Protein TNF and the Glycan-Binding Protein Galectin-9 Is Retained

The utility of the above-delineated strategy of LbL-coated vaterite nanoparticles for protein-based therapeutic DDS
strategies hinges on the release of proteins that have fully retained functional bioactivity. To delineate the suitability of vaterite NPs in this respect, the key immune effector protein TNF was first loaded into vaterite NPs and subsequently released from these particles by overnight incubation at tumor microenvironmental pH or immediate dissolution with EDTA at pH 7.4. Importantly, treatment of tumor cell line FaDu with this vaterite-released TNF significantly upregulated expression of ICAM-1, a protein known to be a target of TNF, approximately threefold (Figure 5C). This upregulation of ICAM-1 expression by TNF released from vaterite particles was similar to that observed upon treatment with the recombinant source TNF (Figure 5C,D).

Treatment of FaDu cells with Gal-9 incubated at the synthesis pH of vaterite triggered a rapid increase in external phosphatidyl serine (PS) (Figure 5F). This upregulation of PS by Gal9 at pH 5.7 was as high as with the control Gal9 as previously reported by us. Thus, the vaterite particle process for protein encapsulation as defined here indeed enables the retention of the biological activity of Gal9 as well as TNF.

3. Discussion

In this study, we developed a framework for vaterite-based DDS suitable for bioactive protein-based therapeutics. In brief, we demonstrated that in the synthesis process, the use of NaHCO₃ is preferred in order to retain bioactivity due to pH conditions. Further, low pI proteins load with high efficiencies of over 90% to vaterite particles, whereas for high pI proteins, the use of biocompatible sulfated polymers such as dextran-sulfate is needed to achieve equally high loading efficiency. Importantly, vaterite can be effectively stabilized using PEs during coprecipitation or upon layer-by-layer coating with a polymer postsynthesis, yielding long-term stability in aqueous conditions. The resultant stable vaterite nanoparticles can release fully bioactive protein-based drugs as demonstrated for lysozyme, the hallmark immunomodulatory protein TNF, and the glycan binding protein galectin-9. This release is controlled on the one hand by the pH of the environment and on the other hand by the kind and number of polyelectrolyte layers deposited by the LbL technology.

Notably, protein-encapsulated vaterite can be exploited both in micro- and nanoparticle applications, with microparticles, for instance, being suitable for local injection as such particles tend to stay where placed, resulting in a sustained drug release. They can also be used for passive targeting of antigen presenting cells since their large size prevents their uptake by the majority of cells except phagocytes. However, microparticles cannot be used for intravenous injection as they are unlikely to cross the biological barriers and may embolize vessels with the same diameter.
A concern for the application of vaterite as DDS has been its instability in aqueous conditions in which it recrystallizes to the thermodynamically more stable calcite, resulting in premature drug release in a non-pH-dependent manner.[22] This undesired transformation was completely suppressed by coating vaterite by certain polyanions such as CMC and PSS. CMC stabilized vaterite better and for a longer time than PSS, which might be due to the coordination effect of carboxylic groups with calcium ions, minimizing the dissolution as well as phase transfer.[23,24] This stabilizing effect of carboxylic groups was also observed when vaterite particles were coated with carboxylic group containing polymers PAA and PMAA.

Loading of proteins at pH 5.7 by means of the coprecipitation process was tolerated by all the bioactive proteins that were tested, with lysozyme even being more stable at pH 5.7 than at pH 7.4 as judged from the higher unfolding temperature. This finding is in line with a previous study in which lysozyme was reported to be more stable at pH 5.[25] Notably, a significant reduction in activity based on reduced unfolding temperature was observed at pH 10.3 for all proteins tested, indicating that this pH is poorly tolerated by most proteins. In line with this, catalase was previously reported to lose about 98% of its activity during calcium carbonate precipitation due to the high alkaline conditions.[16] The unfolding temperature of TNF decreased by 15 °C at pH 10.3 indicating deleterious changes in the protein structure. Such modifications at high pH are likely due to structural changes in asparagine and glutamine, with several deamidations and cleavage of the peptide backbone being reported.[26,27] Likely, these alterations affected the stability of proteins tested here. Therefore, coprecipitation of proteins using sodium carbonate at high pH as described manifold in the literature cannot be recommended for bioactive proteins. For retaining bioactivity, the reaction pH of sodium hydrogen carbonate is better suited.

Figure 5. A) Release of BSA at different pH from bare (dashed lines) and LbL-coated vaterite microparticles (continuous lines) with (CMC/PAAET)$_2$ (flow rate: 0.2 mL min$^{-1}$, time: 2 h), and B) the corresponding vaterite dissolution at the same conditions (bare: black dashed lines, coated: red continuous lines). C) Lysozyme activity at different conditions. D,E) Flow cytometry analysis of ICAM1 expression on the surface of FaDu cells upon treatment with TNF at different conditions. F) Gal9 biological activity as measured by induction of PS-exposure using Annexin-V in FaDu cells at different conditions.
since all the loaded proteins at this pH preserved functional activity in vitro.

Although modifying vaterite particles with a panel of polyelectrolytes yielded highly negatively charged particles, loading of alkaline proteins was only increased with PSS, ECGC, heparin, and sulfonated dextran. Interaction of lysozyme with the sulfonic acid groups of PSS resulted in insoluble precipitates presumably due to the known hydrophobicity of PSS. This electrostatic complexation of lysozyme with PSS and the stability of lysozyme after precipitate formation was studied in more details elsewhere.\(^{[28]}\) ECGC is a tannin that crosslinks proteins by hydrogen bonds and hydrophobic interactions without any covalent or ionic bonds,\(^{[29]}\) but the interconnections alter the nature and function of the protein.\(^{[30]}\) Although such precipitates were not detected with heparin-modified vaterite, which fully dissolved upon vaterite dissolution, the anticoagulant property of heparin raises the concern of using a heparin-modified DDS in blood.\(^{[31]}\) A well-known biocompatible sugar full-dissolved upon vaterite dissolution, the anticoagulant of lysozyme after precipitate formation was studied in more details elsewhere.\(^{[28]}\) ECGC is a tannin that crosslinks proteins by hydrogen bonds and hydrophobic interactions without any covalent or ionic bonds,\(^{[29]}\) but the interconnections alter the nature and function of the protein.\(^{[30]}\) Although such precipitates were not detected with heparin-modified vaterite, which fully dissolved upon vaterite dissolution, the anticoagulant property of heparin raises the concern of using a heparin-modified DDS in blood.\(^{[31]}\) A well-known biocompatible sugar with several known biomedical applications,\(^{[32]}\) sulfated dextran, similarly increased the loading of lysozyme without the formation of protein precipitates, likely due to its hydrophilic backbone. Hence, loading efficiency of alkaline proteins can be increased by premodification of vaterite with several polymers, but based on suitability for biomedical applications choice of dextran sulfate seems most warranted.

In a close-to-physiological flow/pH/temperature setup, vaterite dissolved three times faster at the tumor microenvironmental pH than at physiological pH. Our experimental setup exhibits more the situation in the tumor, where the targeting particles would be fixed in the tissue, e.g., due to active targeting and the blood/interstitial fluid is streaming. In the blood circulation, the relative streaming velocity of blood in respect to the nanoparticles tends to zero, which will furthermore reduce the dissolution due to the depletion of hydrogen ions in the surrounding of the particle. Surprisingly there was no significant difference in dissolution kinetics of MV versus NV (Figure 4E). In addition to the dependence on flow velocity, this points to a control of dissolution by delivery of hydrogen ions. Notably, vaterite dissolution at body temperature was reduced compared to room temperature, possibly due to a decrease in solubility of calcium carbonate at higher temperature.\(^{[33]}\) Indeed, calcium carbonate solubility at 35 °C was 1.16 times lower than at 35 °C and decreased furthermore by 2.9 times up to 90 °C.\(^{[34]}\)

Coating of protein-loaded vaterite particles with alternating layers of polyanions and polycations by means of LbL process not only stabilizes vaterite against undesired phase transformation to calcite, but also enables an additional control of the release rates of proteins. Hereby, unspecific or premature leakage of protein during blood circulation can be reduced further and a continuous slow release in the tumor tissue can be achieved. For instance, BSA release was reduced by ~50% from LbL-coated particles compared to pristine vaterite particles. In addition, the LbL coatings can protect the enclosed proteins for early enzymatic degradation and can be used for defined coupling of target molecules to the particle surface. Nevertheless, the number and material of the layers has to be fine-tuned for each protein and application in order to control the optimal release of proteins.\(^{[36]}\) Also, the choice of polyelectrolyte and the parameter of coating procedure are important not only for biocompatibility, but also to avoid loss of protein during the coating. For example, strong loss of lysozyme-loaded vaterite particles during PSS coating was reported\(^{[15]}\) due to the strong electrostatic interaction of lysozyme with PSS.\(^{[38]}\) This could be avoided in our case by using CMC instead of PSS for subsequent LbL coating.

As a proof of concept, the protein TNF was shown to preserve full functional activity after our loading and preparation process and the subsequent release from vaterite particles. TNF has been of interest for immunotherapy since decades and many studies have been performed to enhance the selectivity.\(^{[16]}\) However, due to its high toxicity at systemic delivery, it has not been applied yet, beyond localized therapy such as isolated limb or liver perfusion.\(^{[36]}\) Encapsulation of TNF as reported here could help to reduce the severe toxicities of TNF encountered in the several phases I and II clinical trials conducted in 1980s and 1990s.\(^{[37,38]}\) In this respect, a pegylated gold nanoparticle was previously reported for the targeted delivery of human TNF-α to solid tumors, an approach that has been clinically evaluated.\(^{[39,40]}\) For vaterite encapsulated TNF, it will be of interest to further delineate whether the tumor-selective pH-driven release of vaterite can be combined with active tumor-targeting to establish TNF-based cancer immunotherapy.

4. Conclusion

We thoroughly assessed all conditions from preparation, protein drug loading, retaining activity, and control of release for the development of vaterite particles as a pH-sensitive drug delivery system for therapeutic protein delivery to tumors. Besides a strategy for preparing small mesoporous vaterite nanoparticles at almost neutral pH, a subset of polyelectrolytes was identified that can stabilize vaterite in aqueous solutions and others that can enhance the loading of proteins with low affinity to vaterite. We identified realistic loading conditions to prevent protein denaturation and developed an in vivo-like release system to mimic (pH) conditions under body-like flow rate. This system provided a more accurate prediction of vaterite dissolution as well as protein release kinetics from coated and uncoated vaterite particles, with the released protein preserving full functional activity in vitro. This study thus provides a framework for future applications development of vaterite-based pH-sensitive DDS for bioactive protein-based tumor therapeutics.

5. Experimental Section

Recombinant Proteins and Reagents: Lysozyme from chicken egg (Lys, ≥90%, Sigma-Aldrich), BSA (≥96%, Sigma Aldrich), and CTX (Hospital pharmacy, UMCG, University of Groningen) were used. TNF was a kind gift from Prof. Harald Wajant, University of Wuerzburg, Wuerzburg, Germany. Carboxymethyl cellulose, sodium salt (90k, Sigma-Aldrich), PSS (70k, Sigma Aldrich), PADOET (Polysciences, Inc.), GelB, heparin (Hep), epigallocatechin gallate (EGCG), dextran (DX), dextran low sulfate (DXLS), DXS, and chondroitin sulfate (Cho) were used. Na2CO3 (Fluka, Germany), NaHCO3 (MERCK, Germany), ethylene glycol (≥98%, ROTH), MES (99%, ROTH), HEPES (99.5%, ROTH), and Tris (99.9%, ROTH) were used. The labeled polymers and proteins were produced by labeling with tetramethylrhodamine.
isothiocyanate (TRITC). Bradford solution (Roti Quant, ROTH) was used.

Preparation of Nano- and Microcarrier Particles: Spherical porous VMPs were prepared as described elsewhere.[41,42] In brief, 3 mL CaCl2·2H2O (1 M) was mixed with 9 mL water after which 3 mL Na2CO3 or NaHCO3 (1 M) was added and the mixture was stirred for 45 s, and then allowed to stand at static conditions for 10–14 min. The particles were washed with water followed by ethanol (99%) and dried at 60 °C.

NV particles were prepared based on a previously reported method.[43] Briefly, 0.33 mM of CaCl2·2H2O and NaHCO3/Na2CO3 were separately dissolved in water solution containing EC (30%, 40%, 50%, 60%, 70%, and 80%), calcium chloride solution (5 M) was added to 25 mL beaker and stirred at 1300 rpm, and then the same volume of NaHCO3/Na2CO3 was quickly added and the mixture was stirred for a given time. Particle’s size was examined at 10, 30, 60, and 120 min of incubation.

Protein Loading: Proteins were loaded to vaterite either by coprecipitation or by postloading. For protein coprecipitation, 3.8 mg of protein was dissolved into 9 mL of water, whereupon calcium chloride (3 mL, 1 M) and NaHCO3/Na2CO3 (3 mL, 1 M) were mixed for 45 s. The solution was allowed to stand for 10–15 min upon which the particles were washed three times with deionized water and freeze dried. For protein postloading, 10 mg of particles was dissolved in Tris buffer (50 × 10−3 M, pH 7.4) and was sonicated briefly. The protein solution was added to give a constant final concentration of 0.25 mg mL−1 for all proteins and a final volume of 1 mL. This mixture was incubated for 30 min at 30 rpm in a rotator (neolab Migge, RM-2M). The amount of protein loaded in the vaterite particles was deduced by measuring protein content remaining in the supernatant using Bradford assay (Roti Quant) following the manufacturer’s protocol.

Analysis of Protein Unfolding: Protein stability at different pH conditions and after release from the particles was evaluated using Tycho (Nanotemper GmbH, Germany), a device for label-free detection of protein quality–purity, concentration based on protein unfolding temperature. In brief, 10 µL protein solution was filled in capillaries and then subjected to increasing temperatures from 35 to 95 °C. The unfolding temperature was exported as automatically analyzed results.

Layer-by-Layer Coating: Nanometer thin layers were assembled on empty or protein-loaded vaterite particles using the LbL technology by alternating deposition of polycations and polyanions based on charge reversal after each layer.[44–46] Briefly, to 50 mg vaterite particles, 0.5 mL of PAOET in HEPES buffer (2 mg mL−1, 0.2 M NaCl, pH 8.0) was added. The mixture was sonicated for 2 min (Proclean 3.0 DSP, Poland) and then incubated for 10 min at 30 rpm. After washing three times with HEPES buffer, 0.33 mM of CaCl2·2H2O and NaHCO3/Na2CO3 were separately dissolved in water solution containing EG (30%, 40%, 50%, 60%, 70%, and 80%), calcium chloride solution (5 M) was added to 25 mL beaker and stirred at 1300 rpm, and then the same volume of NaHCO3/Na2CO3 was quickly added and the mixture was stirred for a given time. Particle’s size was examined at 10, 30, 60, and 120 min of incubation.

Characterization: The morphological examination of vaterite was performed by confocal laser scanning microscopy (CLSM) (Leica, TYPEDMI4000 CS, Germany) and scanning electron microscope (SEM, Phenom XL). Zeta-potential was evaluated using a MALVERN Zeta Potential/Particle Sizer (Model ZEN5600, UK). The mean diameter of the particles was determined by CLSM and disc centrifuge (CPS Instruments, Inc., USA). LbL assembly of PAOET and CMC on vaterite particles was confirmed by determining the zeta-potential.

Analysis of Vaterite Dissolution and Protein Release: Vaterite particles (10 mg) were added to a wheel filter (Rotilabo-Spritzenfilter, pore size 0.22 µm, ø 25 mm, Germany) and tapped to ensure homogeneous distribution on the membrane. The filter was then connected to a syringe (Injeck, 10/60 mL, Germany) containing a buffer solution (Tris pH 7.4/MES pH 6.5/Acetate pH 4.5) and placed in a syringe pump (Harvard apparatus, CAT#53-2226) with a set flow rate. To study the dissolution of vaterite at relevant pH levels, a buffer close to the physiological pH 7.4 found in the peripheral blood,[47] a buffer at the tumor microenvironmental pH of 6.5,[48] and a buffer corresponding to the endosomal pH of 4.5 was used.[44] The filtered buffer was collected at a given time and the amount of dissolved calcium was quantified from the concentration of Ca+2 ions in the solution following a colorimetric method using o-cresolphthalein complexon described by Morin.[49] To study the influence of pH on protein release, 10 mg of BSA-loaded vaterite particles were placed in a low protein binding filter (Acrdis Syringe filter 0.2 µm) as the previous filter retained a significant amount of protein on the membrane. The particles were then exposed to buffers with different pH values at a constant flow rate (0.2 mL min−1) and the release of BSA was quantified.

Lysozyme Activity Assay: Lysozyme activity was evaluated following the enzymatic assay of lysozyme (EC 3.2.1.17) by Sigma-Aldrich. Briefly, 3 mL of Micrococcus lysodeikticus suspension (0.15 mg mL−1 in potassium phosphate buffer, pH 6.2) was transferred to a quartz cuvette. Then, 100 µL of buffer or lysozyme solution of 8.33 µg mL−1 (control, incubated at pH 5.7 for 30 min, or released from vaterite particles) was added and the absorbance at 450 nm was noted every 2 min for 10 min.

Functional Activity of Gal9 and TNF at Low pH and after Release from Vaterite: FADU cells were plated in a 24-well plate at 5 × 103 cells per well and preincubated for 24 h in DMEM supplemented with 10% foetal calf serum. For treatment with TNF, cells were subjected to recombinant TNF, TNF released from vaterite (using 150 × 10−3 M MES buffer at pH 6.5 or EDTA 30 × 10−3 M for 24 h), and empty particles dissolved under the same conditions as a control 24 h. For analysis of ICAM-1 expression, cells were harvested after overnight incubation and stained with APC-conjugated ICAM-1/CD54 antibody (clone 1H4) or isotype mouse IgG2bAPC (vendor) for 1 h at 4 °C. After antigen staining, cells were rinsed, resuspended in PBS, and evaluated for ICAM-1/CD54 expression by flow cytometry (Cytoflex, Beckman Coulter). For treatment with Gal9, FADU cells were detached and treated for 30 min with recombinant Gal-9, rGal-9 incubated in Mes pH 5.7 for 30 min, and Mes pH 5.6 as a control. Subsequently, cells were washed and stained with Annexin-V in Ca2+ buffer, whereupon PS exposure was evaluated by flow cytometry.

Supporting Information
Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest
The authors declare no conflict of interest.

Data Availability Statement
Research data are not shared.

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