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# Impact of bacteria on aggregation of crystalline and amorphous components of infectious urinary stones

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ABSTRACT

Infectious urinary stones consist of an agglomerate of bacteria, highly crystalline struvite, and poorly crystalline and amorphous precipitate (PCaAP). The paper describes an experimental study on the agglomeration of PCaAP in the presence and absence of bacteria of the *Proteus mirabilis* species, in which blind studies are done with bacterial macromolecules with and without lipopolysaccharide. The formation of struvite is avoided by using Mg-free artificial urine. Optical microscopy methods and zeta potential measurements both confirm that the bacterial lipopolysaccharides are the key factors promoting agglomeration, besides the bacterially induced pH change which leads to precipitation in the first place.

## 1. Introduction

Urinary stones are the deposits forming in the urinary tract from substances occurring in it naturally or pathologically. In recent years, an intense increase in the incidence of this disease has been observed in the populations living in highly developed countries, which affects up to 20% of the population, depending on the studied geographical region [1–3]. The causes of the majority of urinary stones are metabolic disorders and improper diet. These stones called metabolic stones constitute up to 70% of all urinary stones [4], and are composed mainly of calcium oxalate and calcium phosphate, in a smaller percentage they are formed from cystine or uric acid. Another type of urinary stones are infectious stones, which constitute from 10 to 30% of all types of stones diagnosed in humans [5,6]. The formation of these stones is associated with a urinary tract infection caused by urease-producing bacteria. These bacteria include such species as: *Proteus*, *Klebsiella*, *Staphylococcus*, *Morganella*. However, the studies show that in 70% of cases, the formation of infectious urinary stones is caused by *Proteus mirabilis* [7,8], while in the remaining 30% of cases, microorganisms of the species *Pseudomonas*, *Klebsiella*, *Providencia*, *Serratia* or *Staphylococcus* are isolated [9]. Like all of these the bacteria, *Proteus mirabilis* have the ability to produce urease – the enzyme that decomposes the urea naturally present in the urine of a healthy person. As a result of this decomposition and cascades of subsequent chemical reactions, the conditions are reached in which solid phases that give rise to infectious urinary stones are formed. These reactions are described in detail in literature [9–13].

The solid phases are mainly magnesium ammonium phosphate hexahydrate ($MgNH_4PO_4{\cdot}6H_2O$) called struvite, which occurs in highly crystalline form [14–16] and whose crystallization is accompanied by the formation of poorly crystalline [15] and amorphous [16] solid phases. Literature data indicate that carbonate apatite, $Ca_{10}(PO_4)_6CO_3$, (CA) and hydroxylapatite, $Ca_{10}(PO_4)_6(OH)_2$, (HAP) [16,17] may be classified as the poorly crystalline phases. It should be noted that the groups $CO_3^{2-}$, $OH^-$ and $PO_4^{3-}$ in CA and HAP can be substituted with various anions thus giving non-stoichiometric forms of CA and HAP. As shown by the results obtained by us [17], such non-stoichiometric forms may be, for example, calcium sodium phosphate carbonate hydroxide, $Ca_9Na_{0.5}(PO_4)_{4.5}(CO_3)_{1.5}(OH)_2$, and chlorapatite, $Ca_{10}(PO_4)_6Cl_2$. The amorphous phases that are components of infectious urinary stones include: amorphous calcium carbonate (ACC), amorphous calcium phosphate (ACP), and/or amorphous carbonated calcium phosphate (ACCP) [16–18]. All of these phases will be called poorly crystalline and amorphous precipitate (PCaAP). A thorough analysis of these phases appearing in artificial urine is presented in [17].

As mentioned above, struvite occurs in the form of crystals with a well-defined habit, most often a coffin-like habit (Fig. 1, arrow 1). The sizes of these crystals reach up to 100 μm along the crystallographic axis *b*. The remaining poorly crystalline and amorphous phases (PCaAP) differ significantly from the struvite crystals as shown in Fig. 1, arrow 3. As follows from this figure, PCaAP (arrow 3) does not show visible

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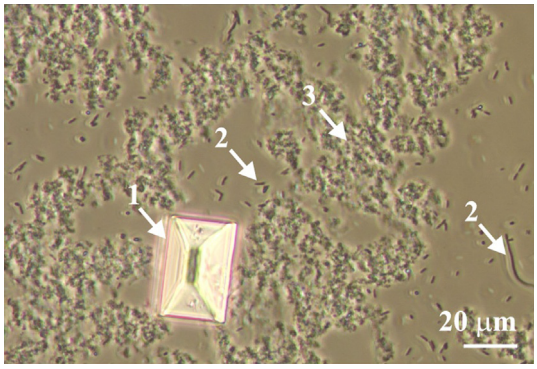


**Fig. 1.** Struvite (arrow 1), bacteria *Proteus mirabilis* (arrow 2) and PCaAP (arrow 3) in the sample of artificial urine. The image was obtained under an optical light microscope with transmitted light. Figure reprinted from [17].

crystalline features and occurs in the form of aggregates without a particular organization [16–18].

Usually, the formed solid phases, struvite and PCaAP, are small enough (tens of micrometers) to be excreted out the urinary tract with urine, without causing damage to the epithelial cells. The infectious urinary stone becomes large not because of single crystal growth, but because of the agglomeration of large amount of relatively small crystals (tens of micrometers) of struvite with small deposits of PCaAP. The aggregation may quickly lead to large stone formation because it occurs quickly, in seconds. The growth of crystals takes place much slower, besides, single crystals are easily removed from the urinary tract and their formation does not lead to the creation of urinary stones. Therefore, the aggregation is supposed to be one of the primary causes of urinary stone formation. It is known from literature that urinary stones contain highly aggregated crystals [19]. Aggregated crystals can be inside not only infectious urinary stones but also metabolic stones, which are built mainly from oxalates and phosphates [19]. Additionally, bacteria may also aggregate with one another and also with struvite and PCaAP. So aggregated bacteria with struvite and PCaAP can be built-in into the structure of the stone [20].

The agglomeration process of struvite, PCaAP and microorganisms is investigated in Ref. [21]. However, it should be noted that in Ref. [21] PCaAP was called CA (carbonate apatite), because at the time when this work was being carried out, we did not know the exact composition of this precipitate and on the basis of literature data (for example Refs. [9,10,18]), we assumed that the resulting precipitate is CA. Now, after the research presented in Ref. [17], our knowledge about the phase composition of the resulting precipitate is greater and hence the name change to PCaAP.

On the basis of the results presented in Ref. [21] it can be concluded that from the three components: struvite, CAaAP (CA in Ref. [21]) and microorganisms, CAaAP has the greatest ability to aggregate. In the present study we evaluate the influence of *Proteus mirabilis* (*P. mirabilis*) on the aggregation of CAaAP in the context of formation of infectious urinary stones. *P. mirabilis* was chosen for analysis because this species has been isolated from human infectious urinary stones in 70% cases [7,8]. In the present study CAaAP aggregation is characterized by cross-sectional area and zeta potential. The aim of the present study is also to analyze the aggregation of CAaAP in the presence of bacterial macromolecules obtained from *P. mirabilis* culture with lipopolysaccharide (LPS) and without it. LPS is the main constituent of the outer membrane of bacteria such as *P. mirabilis* and has crucial role in protection of bacteria from adverse environmental conditions.

## 2. Materials and methods

### 2.1. Preparation of artificial urine, suspension of bacteria and their cellular components

Usually, artificial urine used for various experiments is made of the following components [22], with concentrations (g/l) in brackets: $CaCl_2{\cdot}2H_2O$, calcium chloride dihydrate (0.651), $MgCl_2{\cdot}6H_2O$, magnesium chloride hexahydrate (0.651), NaCl, sodium chloride (4.6), $Na_2SO_4$, sodium sulfate (2.3), $KH_2PO_4$, potassium dihydrogen phosphate (2.8), KCl, potassium chloride (1.6), $NH_4Cl$, ammonium chloride (1.0), $Na_3C_6H_5O_7$, trisodium citrate (0.65), $Na_2C_2O_4$, disodium oxalate (0.023), $CO(NH_2)_2$, urea (25.0), $C_4H_9N_3O_2$, creatine (1.1) and tryptic soy broth (10.0). Such a composition of artificial urine is widely accepted in literature [23,24]. In the present study we focus on the formation of PCaAP, therefore, the composition of artificial urine was modified in such a way that magnesium chloride hexahydrate ($MgCl_2 6H_2O$) was not added. This is a limitation in our experimental setup, however, this is related to the fact that the presence of magnesium in artificial urine causes struvite precipitation, what is undesirable as struvite would be able to disturb the spectrophotometric and zeta potential measurements. Various modifications of the composition of artificial urine are often encountered due to the specifics of the conducted research; without such modifications, some research would not be possible at all (for example Ref. [25]).

The artificial urine of such a modified composition (Mg-free) was prepared by dissolving chemicals (Sigma Aldrich) of reagent-grade purity in distilled water. The further course of action with artificial urine was the same as the procedure described in Ref. [21]. This means that artificial urine was filtered using a membrane filter with pore size of 0.2 μm. It was stored for a maximum of 48 h at 4 °C.

*P. mirabilis* strain was isolated from human urinary stone. Before the crystal growth experiment, the bacteria were maintained on a slant of tryptic soy agar overnight at 37 °C and then suspended in artificial urine to the concentration of $5{\cdot}10^5$ CFU per ml (the abbreviation CFU denotes colony forming unit). Crystallization in artificial urine occurs after addition of the suspension of bacteria and incubation at 37 °C.

Bacterial macromolecules were isolated from the artificial urine which was incubated with the bacteria, as described above. After 24 h of incubation at 37 °C, the urine was centrifuged at 8000 g, at 4 °C for 30 min, to remove bacteria and solid phases from the solution. Then, in order to properly dispose of bacterial cells, the urine was filtered through a filter with pore size of 0.2 μm. The filtered urine was placed in a dialysis sack and dialyzed against distilled water for 24 h at 4 °C. The aqueous solution of bacterial macromolecules remaining in the bag was freeze-dried and used in an appropriate concentration in experiments. That way a mixture of all the bacterial cell components both protein and LPS was obtained. Bacterial macromolecules without LPS were prepared by purification of the sample on Pierce™ High Capacity Endotoxin Removal Spin Columns (Thermo Fisher Scientific). These columns have modified polylysine on their surface which binds with high affinity lipopolysaccharides, as specified by the manufacturer, up to 10 000 EU/ml (1 EU/ml ≈ 0.1 ng/ml). A sample of a concentration of 2 mg/ml in sodium phosphate buffer (pH 7.3) was added to the column and incubated upon gentle stirring, for 1 h at 4 °C. After this time, the sample was recovered by centrifugation, a LPS binding step was repeated three times to achieve the lowest possible LPS concentration (less than 5 EU/ml).

The formation process of PCaAP and its aggregation was also investigated in the presence of bacterial macromolecules with LPS and without it. In this case, Mg-free artificial urine was also used. In the presence of bacterial macromolecules, the precipitation process occurs after the gradual addition of 1.2 M aqueous ammonia solution. The aqueous ammonia solution is added in portions, not continuously. Such an addition causes an increase in pH and the concentration of the ammonium ions. In other words, the addition of aqueous ammonia