

Response to Reviewer 2 Comments

Comments and Suggestions for Authors: The authors treat an interesting problem. Consequently, any advancement in the understanding of it I consider as being of huge interest both from a theoretical point of view as well as for design of medical treatments of the mentioned health problem.

Summarizing, the topic of analysis I consider as highly interesting. The authors have to formulate however more precisely what they have done and in which respect it can be of interest for the analysis of the formation of kidney stones. I recommend major revision.

First of all, we would like to thank the reviewer for all comments and suggestions, which we have given careful consideration. In this work we evaluated the possibility of non-classical crystallization of CaOx via PNC and dense liquid precursors formed during the pre-nucleation stages in the presence of PAA. We also address the effects of PAA on the selection of CaOx phases and morphology of the precipitates formed in the post-nucleation stages. At the same time, we propose an in vitro model that allows studying the effect of acidic macromolecules on CaOx crystallization and to study the inhibitory role played by these macromolecules in the formation of kidney stones.

In this work, the presence of polynuclear ionic complexes (PNC) in pre-nucleation stages was determined by free Ca^{2+} ions measurement through ISE and by conductivity measurements. The conductivity values are the result of the contribution of all ionic species in the system. Here, the concentration of all ionic species in the solution was calculated using the Kohlrausch's law on the mobility of independent ions, determining the molar conductivity of the ionic complexes formed and the difference between the measured conductivity and the theoretical one calculated for the control runs assuming an ideal solution. Then, we determined the concentration of oxalate ions bounded to PNC and the concentration of free oxalate ions in solution for each essay. This methodology has been used by Ruiz-Agudo et al (2017) as it is indicated in ref. #14. Indeed, our experimental results showed a perfect match between the concentration of bound Ca^{2+} and oxalate ions of the ionic complexes with a $\text{Ca}^{2+}/\text{C}_2\text{O}_4^{2-}$ mole ratio of 2 determined using conductivity and the calcium ion selective electrode (Figure S1). These results strongly support our claim that the ionic species present in solution at the pre-nucleation stage are indeed PNC.

All results of the titration essays show a significant difference between the dosed and free Ca. In Figure 1, it is shown that such a difference is evident from the very beginning of the titration essay, meaning that this effect occurs in undersaturated and supersaturated conditions both in the presence and absence of PAA. This enables to disclose that even in the very early stage of the experiments there is an interaction (bonding) between Ca^{2+} ions and $\text{C}_2\text{O}_4^{2-}$ ions, resulting in the formation of multi-ion associates that results in the reduction of the free Ca^{2+} concentration, a reduction that cannot be accounted for by the formation of ion pairs as shown for the case of calcium carbonate. This suggests that the CaOx crystallization occurs through a non-classical crystallization pathway. Indeed, in this work, entities from 20 to 400 nm in size were detected by DLS and TEM from samples collected 250 s before nucleation starts, which would be associated with a non-classical multistage crystallization that involves the participation of large PNC aggregates and dense liquid precursor phases. This would indicate that nucleation is preceded by the aggregation of the above mentioned entities.

TEM analysis showed that these entities formed in the presence of PAA are ungrouped and only occasionally they showed a close contact between them, contrary to what was observed in the control trials. These results suggest that PAA induces electrostatic stabilization, which was corroborated by the Z potential results. Electrostatic stabilization led to the delay in the initiation of nucleation as seen in figure 1, where the nucleation time was shifted from 680 s, without additive, to 980, 1360 and 1850 s in the presence of PAA at 10, 50 and 100 mg/L, respectively. On the other hand, PAA early selects COD hydrate, a thermodynamically less stable crystal phase, that appears to be less active in the formation of kidney stones. Therefore, with this in mind, a decrease in acidic macromolecules synthesized at the renal tubule level could play a key role in the formation of kidney stones and/or the incorporation of synthetic acidic macromolecules in the urinary tract could have a beneficial role in preventing this pathology. This is further

clarified in the revised version of the manuscript, where we also further indicate the importance of our results in the development of kidney stones (see below).

Point 1: The authors should clearly distinguish which nucleation process they consider. So, the statement of the authors “we show how acidic macromolecules influence the dynamics and kinetics of CaOx crystallization by exerting control on the pre- and post-nucleation stages by delaying the crystallization of solid phases and allowing the stabilization of PNCs” I consider as only partly correct. And the main problem which has to be obviously resolved is why (as noted by the authors) “only COM and COD are found in urine, whereas COM, the most thermodynamically stable form, is the main inorganic crystal found in 42 kidney stone”. As it seems to me this problem is even not touched.

Response 1: We thank the Reviewer for this observation and critical issue. In this work, we considered that the nucleation process occurs through a non-classical multistage crystallization pathway, which involves the formation of PNC in unsaturated solutions, determined by free Ca^{2+} by ISE and conductivity measurements and the participation of large PNC aggregates and dense liquid precursors (PILPs) detected in supersaturated solutions (see also our response above). In turn, PAA influences the crystallization dynamics favoring and stabilizing the formation of PILPs, which leads to a delay in nucleation. On the other hand, PAA influences the precipitated phases, selecting the hydrated COD form over the COM, which could have implications in the pathophysiology of kidney stones, since COD is suggested to be less active in the formation of kidney stones, which is possibly related to the difference in solubility of both hydrates. This is now discussed in the revised version of the manuscript. Please see pages 9, 10 and 14, in the Results and Discussion section of the revised version of the manuscript.

Point 2: Dillman and Meier Ref.5 analyze condensation of gases. Why this paper is cited and not papers dealing with segregation and crystallization in solutions?

Response 2: We completely agree with the reviewer's comment. The citation of this reference was corrected in the revised manuscript. Please see page 16, in the References section.

Point 3: The authors state: “Biogenic mineral crystallization processes always involve the stages of nucleation and crystal growth [8]”. Question: Do the authors assume that this is a specific feature of biogenic materials?

Response 3: This phrase was intended to highlight that any crystallization process involves nucleation and crystal growth, regardless of its nature. In this regard, the pertinent correction was made in the main text of the manuscript in order to avoid confusion and not to assume that it is a specific and unique characteristic of biogenic materials. Please see lines from 38 to 39 in page 1, in the Introduction section.

Point 4: What is the meaning of the statement “Crystallization pathway that involves a liquid-liquid phase separation and the formation of a dense liquid phase in supersaturated solutions.”

Response 4: This statement aimed to reinforce the fact that during the pre-nucleation stages, the participation of precursors with a liquid-like character can take place. Such liquid-like (or dense liquid) precursors form via liquid-liquid phase separation and typically display sizes from tens of nanometers to a few micrometers, as demonstrated in various crystal systems (see relevant refs. cited in our manuscript). Such entities form in supersaturated solutions, a condition that favors a liquid-liquid phase separation, giving rise to droplets of a dense liquid phase in the solution. Thus, during the crystallization pathway of different minerals, the formation of a liquid precursor can occur in the presence or absence of additives, and subsequently the nucleation of a solid amorphous precursor phase can take place within such a dense liquid by water expulsion and densification (an entropy-driven process). To clarify this statement, we have rephrased lines 69 to 72 in page 2, of the Introduction section.