Synthesis of Mg and Sr substituted hydroxyapatite following a design of experiments approach

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INTRODUCTION: As the basis of bone mineral, hydroxyapatite (HAP) has been widely studied for orthopaedic applications. For example, HAP has been used to improve the outcome of spine surgery, both as an osteoinductive coating for fusion cages, or as the basis for synthetic bone grafts. However, biologic HAP is not stochiometric, and presents on its structure different ionic species besides Ca^{2+}, PO_4^{3-} and OH^-, each one with different roles in bone development, remodelling, and healing. Synthesizing HAP with different ionic substitutions and, consequently, closer to its biologic counterpart leads to HAP-based implants with enhanced biologic performance. Mg^{2+} and Sr^{2+} are two interesting possible substitutions, due to their individual abilities to, respectively, stimulate osteoblast activity and mineralization and inhibit osteoclast activity and bone resorption [1]. Moreover, research has also shown that HAP synthesis is optimal when pH is within a 10 – 12 range [2]. As such, it is of interest to study how Mg^{2+} and Sr^{2+} might interact with one another, and what formulation and synthesis conditions might lead to substituted HAP (sHAP) with an optimized biological response. The goal of this work is to use design of experiments (DoE) to study how different initial concentrations of Mg^{2+}, Sr^{2+} and ammonia solution can affect the synthesis and biological performance of sHAP with different substitution degrees, and to use these results to assess which conditions will lead to sHAP with the most enhanced osteogenic potential.

METHODS: sHAP with different substitution degrees was synthesized by adding drop-by-drop a 500 ml H_3PO_4 solution to a 500 ml solution containing the Ca^{2+}, Mg^{2+} and Sr^{2+} precursors, as well as a specific volume of ammonia solution 28%, at 40 °C. Then, the final suspension was left maturing overnight at 37 °C. $Ca(OH)_2$, $MgCl_2 \cdot 6H_2O$ and $Sr(NO_3)_2$ were used as cationic precursors. All sHAP samples were synthesized to have a theoretical (Ca+Mg+Sr)/P ratio equal to 1.66. The synthesis of sHAP was studied using a full factorial DoE with three centre points. Table 1 describes the factors studied. All samples had their composition analyzed by ICP-OES, and their crystal phases analyzed by XRD. DoE analysis was performed using the software *Minitab 20.0*, using as design responses Ca/P ratio, (Ca+Mg+Sr)/P ratio, Mg %, Sr %, and HAP phase %.

Table 1 - Factor description

Factor	Description	-	0	+
A	Mg substitution degree (%)	5	7.5	10
B	Sr substitution degree (%)	5	7.5	10
C	Ammonia solution 28% volume (ml)	15	32.5	50

RESULTS: The main effects of each optimized DoE model, including the average response β_0, as well as their respective summary of fit results are all presented in table 2. Higher substitution degrees appear to decrease Ca/P, (Ca+Mg+Sr)/P and the HAP phase %, although higher volumes of ammonia solution help to mitigate this. For the substitution degrees studied, the incorporation of Mg^{2+} and Sr^{2+} into the structure of sHAP seem to be independent of one another, although the incorporation of Mg^{2+} is facilitated by the presence of higher volumes of ammonia solution. All models pass all four summary of fit tests, and as such, can be considered as valid models for the region in study.

Table 2 - Effects of main factors and interactions, and summary of fit of optimized DoE models (Box-Cox transformation of exponent λ)*

Model	β_0	A	B	C	AB	AC	BC	R^2	Q^2	Validity	Reproducibility
Ca/P	1.3618	-0.0525	-0.0550	+0.0150	--	--	--	96.56	93.85	98.42	89.29
(Ca+Mg+Sr)/P	1.5736	-0.0300	-0.3500	+0.0550	--	--	--	90.64	74.00	81.42	88.27
Mg % * λ = 4	3.803	+3.109	--	+3.171	--	+2.632	--	99.11	97.26	73.28	99.16
Sr % * λ = 1	6.8609	--	+1.9575	--	--	--	--	99.92	99.88	73.93	99.95
HAP phase % * λ = 9	11.676	-4.811	-1.313	+1.566	--	--	--	81.14	54.44	87.95	73.73

DISCUSSION: Using DoE, it was possible to develop different valid mathematical models able to predict how the synthesis conditions can affect the quality of sHAP, as well as how those conditions affect the incorporation of Mg^{2+} and Sr^{2+} into the HAP structure. The next step will be to assess *in-vitro* the biologic performance of each sHAP sample by indirect contact on hTERT-MSCs Y201 [3]. Two new DoE models will be formulated, using as design response the metabolic activity at day 14, measured by resazurin reduction assay, and ALP activity at day 14, measured by p-nitrophenyl phosphate colourimetry. The *in-vitro* characterization models, allied with the previous ones, will determine which synthesis conditions result in sHAP with optimized osteogenic potential.

SIGNIFICANCE/CLINICAL RELEVANCE: Slow or improper osteointegration is still a common issue that can lead to longer recovery periods after spinal fusion surgery and/or implant failure, with 1 in 5 patients requiring reoperation within 4 years of initial surgery [4]. The use of biomimetic materials can enhance osseointegration. Moreover, the use of DoE facilitates the optimization of biomaterials, by efficiently predicting which synthesis conditions result in materials with better biologic performance.

REFERENCES:
[1] K. Lin, J. Chang, Hydroxyapatite (Hap) for Biomedical Applications, Woodhead Publishing (2015) 3-19.
[2] D. Ham, et al., Journal of the Korean Physical Society 75(7) (2019) 514-518.
[3] S. James, et al., Stem Cell Reports 4(6) (2015) 1004-1015.
[4] T. M. Irmola, et al., Spine 43(4) (2018) 295-301.

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