Three-dimensional arrangement of β-tricalcium phosphate granules evaluated by microcomputed tomography and fractal analysis

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The macrophysical properties of granular biomaterials used to fill bone defects have rarely been considered. Granules of a given biomaterial occupy three-dimensional (3-D) space when packed together and create a macroporosity suitable for the invasion of vascular and bone cells. Granules of β-tricalcium phosphate were prepared using polyurethane foam technology and increasing the amount of material powder in the slurry (10, 11, 15, 18, 21 and 25 g). After sintering, granules of 1000–2000 μm were prepared by sieving. They were analyzed morphologically by scanning electron microscopy and placed in polyethylene test tubes to produce 3-D scaffolds. Microcomputed tomography (microCT) was used to image the scaffolds and to determine porosity and fractal dimension in three dimensions. Two-dimensional sections of the microCT models were binarized and used to compute classical morphometric parameters describing porosity (interconnectivity index, strut analysis and star volumes) and fractal dimensions. In addition, two newly important fractal parameters (lacunarity and succolarity) were measured. Compression analysis of the stacks of granules was done. Porosity decreased as the amount of material in the slurry increased but non-linear relationships were observed between microarchitectural parameters describing the pores and porosity. Lacunarity increased in the series of granules but succolarity (reflecting the penetration of a fluid) was maximal in the 15–18 g groups and decreased noticeably in the 25 g group. The 3-D arrangement of biomaterial granules studied by these new fractal techniques allows the optimal formulation to be derived based on the lowest amount of material, suitable mechanical resistance during crushing and the creation of large interconnected pores.

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1. Introduction

Several types of biomaterials have been proposed that can be used to fill bone defects in implant dentistry, orthopaedics or plastic surgeries. The use of morcelized bone particles or synthetic biomaterial granules has been recognized for decades, to provide templates for osteoconduction [1,2]. Synthetic biomaterials such as ceramics offer the possibility of being produced in large amounts and of being prepared with a micro-and/or macroporosity [3,4]. The geometry of the gathered material is considered as a critical parameter to favor bone formation [5]. This has been particularly studied for designing porous scaffolds because an interconnected porosity is required to allow the migration of fluids and cells through the three-dimensional (3-D) microarchitecture [6–8]. However; little is known about the 3-D disposition of the biomaterial granules implanted in a grafted site. β-tricalcium phosphate (β-TCP) is a well-known ceramic with bioactive properties [9,10]. When prepared by the polyurethane-foam technique, the shape of β-TCP granules can be controlled by scanning electron microscopy (SEM) or X-ray microcomputed tomography (microCT) [11]. The spatial disposition of β-TCP granules according to their shape has never been studied. It is essential to develop granules with the best adapted shape that would favor the invasion of vascular growth bringing bone cells and allowing the diffusion of nutrients in the very center of the grafted area. MicroCT allows a quantitative evaluation of the material volume and porosity in three dimensions but the method cannot provide a good description of pore interconnectivity and geometry. Several algorithms have been described to evaluate the interconnectivity of marrow spaces in bone tissue (i.e. the porosity of trabecular bone) on two-dimensional (2-D) sections [12,13]. The fractal dimension can evaluate the complexity of the material to fill the reference space and is helpful in the study of porous objects such as bone.
or materials [16,17]. Recently, new fractal parameters have been described (lacunarity and succolarity) to evaluate the heterogeneity and connectivity of the drainage patterns within an object [18–20]. Lacunarity is a concept introduced by Mandelbrot to describe the nature of gaps, voids or pores within texture images [21]. Succolarity is another concept proposed by Mandelbrot and represents the degree of percolation of an image (i.e., how a fluid can flow through an image) [21]. In the present study, we have used granules of \( \beta \)-TCP with different shapes by varying the amount of the slurry in the polyurethane foam. Granules were placed in test tubes and allowed to settle. The 3-D arrangement of the granules was evaluated by microCT, and 2-D sections were used to compute fractal dimensions, lacunarity and succolarity in order to better characterize the porosity created between the granules.

### 2. Materials and methods

#### 2.1. Preparation of \( \beta \)-TCP granules

Granules of \( \beta \)-TCP were prepared by the polyurethane foam technology as previously described [11,22]. Different volume fractions were investigated: 10, 11, 15, 18, 21 and 25 g of \( \beta \)-TCP powder were mixed with distilled water to produce six types of slurries. They were used to impregnate 1 g cubes of polyurethane foam under vacuum. The blocks impregnated with the slurries were then dried in an oven and heated at 800 °C to completely burn the organic polymer foam. Then the 3-D scaffolds of \( \beta \)-TCP were sintered at 1200 °C; granules were obtained by crushing the blocks in an alumina mortar and the 1000–2000 \( \mu \)m fraction was collected by sieving. This size of granule is the most commonly used for dental and implant surgery. The frequency distribution of the granules’ size (measured by SEM) was always found to follow a log-normal distribution as expected (data not shown). This method provided a series of granules that differed in shape, according to the amount of \( \beta \)-TCP in the slurry. The shape of the granules was controlled by SEM before use.

#### 2.2. MicroCT

The Skyscan 1172 X-ray microcomputed tomograph (Bruker microCT, Kontich, Belgium) was used in the cone beam acquisition mode. Granules of the different grades were placed in polyethylene test tubes (5 mm in diameter, 4 cm\(^3\) of granules in each tube). The tubes were gently agitated to allow granules to settle (Fig. 1). For each series, three test tubes were prepared and microtomographed at 80 kV, 100 \( \mu \)A with a 0.5 mm aluminum filter. The pixel size was fixed at 4.95 \( \mu \)m and a 0.25° rotation angle was applied at each step. 2-D cross-sections of the series of projections images. They were used to prepare 3-D models of the granule arrangements by using the software provided by Bruker microCT (CTvol for surface rendering and CTvox for volume rendering). The fractional amount of material (\( \text{Mat.V/V} \)) and the porosity (\( \text{Po.V/V} = 100 - \text{Mat.V/V} \)) in \% were determined by the CTAn software. The fractal dimension (\( D_{fr} \)) of the granules arrangement was determined in three dimensions by the Kolmogorov “cubic box counting” method, which is an extrapolation of the 2-D method (see below) to the 3-D space. The side of the cube ranged from 2 to 100 pixels.

For determining lacunarity and succularity, 2-D sections were re-sliced from the 3-D models: two sagittal sections (separated by 200 \( \mu \)m) were obtained, and the procedures were done in orthogonal planes. So, for each test tube, four sections were obtained and binarized.

![Fig. 1. Methods used to prepare the images suitable for 2-D and 3-D analysis. (A) Granules of \( \beta \)-TCP are placed in a test tube and are allowed to settle. (B) The test tube is analyzed by microCT. (C) The 3-D model is reconstructed and re-sliced in two orthogonal directions to provide the 2-D images suitable for analysis after binarization (D).](image-url)
“gliding box” method [18,28]. The technique was recently adapted to porous biomaterials (see details of the algorithms in Ref. [29]). Briefly, a square box of side \( \epsilon \) was glided along all possible directions of the image. The total number of flooded pixels counted during this process was calculated. The procedure was repeated by gradually increasing the size of boxes. The total number of flooded pixels was defined by a mass distribution \( n(M, \epsilon) \). By dividing this number by the total number of boxes of size \( \epsilon \) the probability distribution of \( Q(M, \epsilon) \) was obtained and corresponded to the frequency of the number of occupation of a box of mass \( M \) and size \( \epsilon \). Local lacunarity (\( \delta \)) for a box size \( \epsilon \) was defined by the ratio between the second moment and the square of the first moment. A log–log graph of \( \log(\delta) \) and \( \log(\epsilon) \) was used to determine \( \delta \) from the slope of the regression line. Lacunarity (\( \delta \)) was measured on the pores, i.e. on the white pixels in Fig. 1D.

Succolarity (\( \sigma \)) was calculated according to de Melo and Conci using a box counting approach on a square image of side \( n \) pixels [19]. Succolarity was calculated in four directions: e.g. from left to right, from right to left, from top to bottom and from bottom to top. Briefly, in a first step, the image was flooded in a given direction, ensuring that all black pixels of the first column were detected. Then all the four connected black pixels were selected until an impenetrable mass of white pixels was encountered. The flooded image was then analyzed using the sliding box method with the size of the box \( t \) ranging from 2 to \( n - 1 \), where \( n \) is the size of the image (i.e. \( t \) is the factor of division of the image) [18]. The number of flooded pixels in the box \( B \) was determined. In a third step, the occupation percentage of the box \( PO(B) \) was calculated as the ratio of the number of flooded pixels and the square size of the box. The “pressure” (\( PR \)) exerted on the boxes (by analogy to the liquid which has flooded the image) is stored in an array of pressures. The pressure increases from line to line (or column to column) along the direction of the flood. Finally, succolarity for a given direction was calculated as then ratio of

\[
\sigma_{(\text{direction},B)} = \frac{\sum_{i=1}^{n} PO(B_i) \times PR(B_i)}{\sum_{i=1}^{n} PR(B_i)}
\]

Values were similarly computed vertically (from top to bottom and bottom to top) and horizontally (from left to right and right to left) for the pores (resp. \( \sigma \)) and \( d \) (resp. \( \epsilon \)) i.e. on the white pixels in Fig. 1D.

2.4. Biomechanical analysis

The compression characteristics of the different \( \beta \)-TCP powders were determined on an Instron 5942 machine with Bluehill software (Instron, Elancourt, France). The powders were placed into the test cell and compacted with a punch that fits with the cell diameter (10 mm in diameter). The maximum load was fixed at 400 N with a 2 mm min\(^{-1}\) displacement. For each type of granule, four measurements were done and averaged to provide the compression curve of each type of \( \beta \)-TCP granule. The compression curve of each type of granule was obtained. The stiffness was automatically provided by measuring the tangent to the curve at the beginning of the deformation. The maximum displacement of the punch was obtained when the load reached 400 N. The work to failure was determined by measuring the area under the curve.

2.5. Statistical analysis

Statistical analysis was performed using the Systat statistical software release 13.0 (Systat Software Inc., San José, CA). All data were expressed as mean ± standard error of the mean. Differences between groups were analyzed by a non-parametric analysis of variance test (Kruskall–Wallis) with the Conover–Inman post hoc test. Differences were considered significant when \( p < 0.05 \). When non-linear relationships were obtained, the equation which best described the points was searched using TableCurve 2D (Systat Inc, release 5.01). The value of the coefficient of correlation \( r \) was calculated using a linear (\( r_{\text{lin}} \)), a \( x^3 \) polynomial regression (\( r_{\text{pol}} \)), a logarithmic (\( r_{\text{log}} \)) or an exponential (\( r_{\text{exp}} \)) equation.

3. Results

3.1. The \( \beta \)-TCP granule morphology

The porosity of the granules was evidenced with the naked eye and confirmed by SEM. The 10 g granules were the most porous with thin walls of sintered \( \beta \)-TCP but they were very difficult to handle due to a high fragility (Fig. 2). In contrast, the 25 g granules were denser, with numerous concave surfaces and rare macropores. The other formulations were associated with intermediate aspects: e.g., the 15 g granules had numerous macropores, thicker walls and they were less friable than the 10 g ones. As previously reported, the inner porosity due to sublimation of the polyurethane foam was always clearly identified in each group of granule. The external surface of the granules showed concave areas with the classical hexagonal pavement due to the surface melting of the grains.

3.2. Biomechanical analysis

The compaction curve of each type of granule appears in Fig. 3A. For granules with the lowest formulation, the curves were exponential. The first domain of the curve corresponds to the collapse of the granules and the deformation is due to their irreversible compression. There was a direct relationship between the maximum displacement of the punch and the type of the granules: the more porous granules were associated with the highest displacement of the punch (\( r_{\text{pol}} = 0.95, p < 0.0001 \) (Fig. 3B). In contrast, the work to failure increased with the density of the granules (\( r_{\text{pol}} = 0.92, p < 0.0001 \) (Fig. 3C) but no significant difference was observed between the first three groups. Stiffness confirmed the previous results and there was a positive exponential relationship between the amount of \( \beta \)-TCP grains used to prepare the granules and this parameter (\( r_{\text{exp}} = 0.97, p < 0.0001 \) (Fig. 3D).

3.3. Morphometric analysis

Videos obtained with the 10 and 25 g granules are provided as online Supplementary Materials. Binarized images of the 2-D sections obtained after re-slicing the 3-D microCT models appear in Fig. 4. Morphometric parameters depicting the spatial arrangement of the granules are provided in Table 1. There was a non-linear negative relationship between porosity and the amount of powder in the slurry (\( r_{\text{pol}} = 0.98, p < 0.0001 \) (Fig. 5A). Porosity was inversely correlated with \( N/F \), which reflected the compactness of the granule shape (\( r_{\text{lin}} = 0.84, p < 0.001 \) (Fig. 5A). Porosity was linearly correlated with Euler–Poincare's number (\( r_{\text{lin}} = 0.96; p < 0.001 \) but not with \( V_{\text{Mat}} \). On the contrary, there was a highly significant and negative log-correlation with \( V_{\text{Mat}} \), meaning that the pore size between the granules was reduced as a function of the amount of powder in the slurry (\( r_{\text{lin}} = 0.81, p < 0.0001 \). There was an exponential correlation between the groups and \( V_{\text{Mat}} \) (\( r_{\text{exp}} = 0.99, p < 0.0001 \) (Fig. 5B). The relationship between \( V_{\text{Por}} \) and the different groups of granules appeared with an inverted U-shape with a maximum for the 15 g group (Fig. 5B). The best-fit equation was a polynomial regression (\( r_{\text{pol}} = 0.50, p = 0.02 \). ICI (reflecting the connectivity of the pores) had a mirror U-shape curve (\( r_{\text{pol}} = 0.88, p < 0.0001 \).
3.4 Fractal parameters

The different fractal parameters according to the amount of β-TCP powder in the slurry appear in Table 2. $D_{3D}$, $D_k$ and $D_{MB}$ regularly decreased as a log function of the amount of material in the slurry. $D_k$ and $D_{MB}$ were linearly correlated ($r_{lin} = 0.94$; $p < 0.001$). $D_{3D}$ was linearly correlated with $D_k$ ($r_{lin} = 0.46$; $p = 0.05$) but the correlation with $D_{MB}$ did not reach significance. Due to the size of the images, which was higher when considering the vertical direction (i.e., top-to-bottom and bottom-to-top analysis), the...
factor of division of the image was higher for the vertical direction (Fig. 6). However, evolution of $r|\$ reached the same nadir, meaning that no preferential orientation of the granules was observed. Similarly, $r_3$ also reached a nadir, either in the left-to-right or right-to-left direction but $r_3$ values were always lower than the corresponding $r|$ values due to the reduced width of the image in the horizontal direction (due to the reduced amount of granule).

For further statistical comparisons, only the average value of $r|$ will be used as $r|$ and $r_3$ were linearly correlated ($r = 0.999; p < 0.0001$). A polynomial relationship was evidenced between $r|$ and the different groups of granule as a function of the amount of powder ($r_{pol} = 0.83, p < 0.0001$) (Fig. 7A). Lacunarity increased from the 10 g group up to the 15 g group and then decreased to reach the lowest value in the 25 g group ($r_{pol} = 0.94, p < 0.0001$) (Fig. 7A). $\delta$ and $r|$ were linearly correlated ($r_{lin} = -0.90; p < 0.001$); $\sigma|$ was negatively correlated with porosity ($r_{lin} = -0.91; p < 0.001$) (Fig. 7B) and $\delta$ was positively correlated with porosity ($r_{lin} = 0.89; p < 0.001$) (Fig. 7C). $D_{\text{HM}}$ ($r_{lin} = 0.74; p < 0.001$) and the Euler–Poincaré number ($r_{lin} = 0.87; p < 0.001$) and highly correlated with $V_{\text{Mat}}$ ($r_{lin} = 0.89; p < 0.0001$) and the Euler–Poincaré number ($r_{lin} = 0.87; p < 0.0001$) (Fig. 7D).

4. Discussion

In this series, changing the amount of $\beta$-TCP powder in the slurry produced granules with considerably different shapes, leading to different types of 3-D arrangement and biomechanical property. Granules prepared with the lowest amount of material were brittle with very thin and fragile branches. In contrast, granules prepared with 25 g appeared more compact and presented fewer branches. The inner porosity due to sublimation of the polyurethane foam was always visible in SEM analysis. This was previously demonstrated and analyzed by microCT and SEM [11]. The free surface of the granules always exhibited the classical paving composed of geometric tiles with a round top corresponding to the melting of the individual grains during sintering. Between the grains, some micropores are known to occur [30] and their number was increased in the lowest formulations (data not shown). The biomechanical behavior of the different types of granule was in accordance with previously published results on the compression
of powders [31,32]. Briefly, the granules occupy the full volume of the test cell and their arrangement depends on their shape. When the punch compacts the 3-D stack of granules, the first step is a rearrangement by sliding and rotation, producing a denser stack. The plastic deformation of the 3-D scaffold of the granules starts when the granules are no longer movable relative to each other. The technique is destructive for the granules, which are crushed when the granules are no longer movable relative to each other. The placement of the punch was maximal in the groups prepared with the lowest concentration and the relationship was non-linear. In contrast, the work-to-failure and stiffness increased non-linearly as a function of the amount of powder in the slurry. It is likely that the 25 g granules which are the more compact are associated with higher compressive values. However, the biomaterial always remains more brittle than bone but the granules are stiff, a condition that favors osteoblast differentiation [33]. A classical recommendation made to surgeons by manufacturers is to gently pack the granules in the grafted area without crushing them, in order to preserve the microarchitecture produced.

MicroCT analysis of the different groups of granules clearly showed the 3-D arrangement. The porosity created between the granules can be evidenced on the 3-D models (see video in the online Supplementary Materials) as well as on the 2-D slices prepared from the stacks. Large voids can be noticed in the different stacks but contacts between the granules are due to the very thin branches obtained in the first groups. MicroCT is now recognized as a very important tool to study the 2-D and 3-D characteristics of porous materials such as bone or bone substitutes [34–37]. The method has been extensively used to describe the microarchitecture of cortical and trabecular bone in metabolic and malignant bone diseases. However, the 3-D algorithms are not fully validated and marked discrepancies have been reported by our group and others when comparing histomorphometry on histological sections and 3-D analysis [38–40]. In the present study, 3-D analysis was used to compute porosity (or reciprocally, the volume fraction of the material) and the fractal dimension \( D_{3D} \) by the box plot in three dimensions. Other parameters which characterize porosity were better explored on 2-D images of a large stack of granules obtained after re-slicing the 3-D models. Robust algorithms such as ICI, star volume, the Euler–Poincaré number and strut analysis were easily applied [13,23,24]. In addition, fractal dimensions obtained by the box-counting technique and the dynamic blankets were used to characterize the granules [14]. The use of newly described fractal parameters such as succolarity and lacunarity were found highly interesting to evaluate porosity [19,41]. These methods were previously found useful in the analysis of continuous porous blocks of materials [29] and were applied here to study the relationships of granules packed within test tubes. This condition mimics what is happening when a bone defect is filled with these types of granular materials and the voids between the

### Table 2

<table>
<thead>
<tr>
<th>Parameter</th>
<th>10 g</th>
<th>11 g</th>
<th>15 g</th>
<th>18 g</th>
<th>21 g</th>
<th>25 g</th>
</tr>
</thead>
<tbody>
<tr>
<td>( D_K )</td>
<td>1.485 ± 0.008</td>
<td>1.464 ± 0.006</td>
<td>1.435 ± 0.005</td>
<td>1.430 ± 0.006</td>
<td>1.426 ± 0.003</td>
<td>1.386 ± 0.005</td>
</tr>
<tr>
<td>( D_{MB} )</td>
<td>1.230 ± 0.006</td>
<td>1.224 ± 0.005</td>
<td>1.186 ± 0.003</td>
<td>1.175 ± 0.002</td>
<td>1.159 ± 0.005</td>
<td>1.133 ± 0.005</td>
</tr>
<tr>
<td>( D_{3D} )</td>
<td>2.157 ± 0.013</td>
<td>2.164 ± 0.001</td>
<td>2.123 ± 0.002</td>
<td>2.114 ± 0.002</td>
<td>2.173 ± 0.003</td>
<td>2.167 ± 0.007</td>
</tr>
<tr>
<td>( \sigma )</td>
<td>0.26 ± 0.001</td>
<td>0.03 ± 0.001</td>
<td>0.02 ± 0.001</td>
<td>0.016 ± 0.000</td>
<td>0.058 ± 0.012</td>
<td>0.085 ± 0.008</td>
</tr>
<tr>
<td>( \delta )</td>
<td>0.008 ± 0.001</td>
<td>0.009 ± 0.001</td>
<td>0.012 ± 0.000</td>
<td>0.015 ± 0.003</td>
<td>0.018 ± 0.001</td>
<td>0.034 ± 0.006</td>
</tr>
</tbody>
</table>

Fig. 5. Morphometric analysis showing the variation of porosity and 2-D descriptors according to the amount of β-TCP in the slurry used to prepare the granules: (A) porosity and N/F; (B) star volume of the pores and material and ICI of the pores.

Fig. 6. Measurement of \( \sigma \) (succularity) in the four directions: \( \sigma_1 \) i.e. from top to bottom; \( \sigma_2 \) bottom to top: \( \square \) (plain line) then \( \sigma_3 \), i.e. from left to right: \( \bullet \) and right-to-left: \( \bigcirc \) (dotted line) in a 3-D arrangement of 10 g granules. The “factor of division” in the abscissa corresponds to the size of the sliding box used for computing \( \sigma \).
granules are rapidly filled in vivo with blood and extracellular fluids. These voids are similar to the porosity obtained in a solid block of 3-D materials and as such can be explored by the same methods. However, the number of papers concerned with the 3-D architecture of particle aggregates is very limited [42]. In the present study, porosity regularly decreased as a function of the amount of powder used to prepare the granules but as previously reported for bone and porous scaffolds, the relationships between material quantity and its 3-D microarchitecture are not linear.

\( V/C3 \) Mat increased exponentially in the different groups of granules but the pores between the granules were correlated by non-linear or complex polynomial functions that depend on the shape of the granules. This is in relation to the presence of branches that can separate granules from each other. The maximal size of the pores was obtained for the 15 and 18 g granules, which had the highest \( V/C3 \) Pore and lowest ICI values.

Lacunarity is known to be influenced by the uniformity of the spatial distribution of the pore density [43]. The maximal \( \delta \) value was reached in the 15 g group, meaning that some large pores are created in the 3-D packages of the granules. On the other hand, the lowest \( \delta \) value was obtained for the 25 g granules, which presented the most regular distribution of the pores, as evidenced by the linear relationship between \( \delta \) and \( V_{Pore} \). Similar relationships have been reported in studies concerned with the porosity of soils [44,45].

Succolarity was extensively evaluated by de Melo in his thesis and related papers [18,19,41]. In this study, \( \sigma \) was calculated on the interconnected pores (i.e., the white pixels appearing on the binarized images of the stacks of granules). Succolarity also offers the advantage of being a direction-sensitive parameter, which can identify preferential directions within a texture [18]. In this study, \( \sigma \) was maximal in the 25 g group composed of dense granules with a very few branches and homogenous pores between the granules. \( \sigma \) was positively correlated with \( V_{Pore} \), but no relationship could be found with \( V_{Mat} \). ICI, which reflects the interconnectivity of the pores (and follows a U curve in the different groups), is also high in the 25 g group. Succolarity is an interesting parameter for porous biomaterials since it can be compared to the accessibility of biological fluids within the grafted site. This parameter offers more interesting data than the classical \( D_K \) parameter used in other studies [42]. Here, we found that the left-to-right and right-to-left values were identical, as were the top-to-bottom and bottom-to-top values. The differences between the horizontal (\( \parallel \)) and vertical (\( \perp \)) values were dependent only on the width of the images and no preferential direction could be evidenced.

5. Conclusion

The interest in these methods is in choosing the best formulation to prepare granules of \( \beta \)-TCP with suitable shape and usable to fill bone defects. To summarize, granules (1) should be prepared with the minimum amount of biomaterial to ensure the fastest resorption rate, (2) should be resistant enough to avoid shrinkage or crushing when the surgeon compresses them with a plunger and (3) should maintain the largest pores to allow invasion by the vascular sprouts. From this study, it is concluded that 15–18 g in the slurry would be the optimal formulation.

Conflict of interest

The authors declare no conflicts of interest.
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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.actbio.2014.09.015.

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