Kinetic deposition rates of Captal® and synthetic HA also support the fact that synthetic HA is less crystalline and more biocompatible than Captal®. We compared the dispersibility of these nanoparticles in Simulated Body Fluid (SBF) by measuring the absorbance of the dispersion versus time (**Figure 23**). We normalized the absorption values using the initial absorption value of each sample and the deposition curves showed that Captal® has a quicker deposition rate, meaning that Captal® has less capacity of dispersing in simulated body fluid. This lower interaction between Captal® and a biological fluid has an immediate consequence, which is that Captal® is less biocompatible than synthetic HA. Apart from this, it can also be inferred that Captal® has a more crystalline structure that lowers the ionic and non-ionic interaction energies between Captal® and its surroundings.

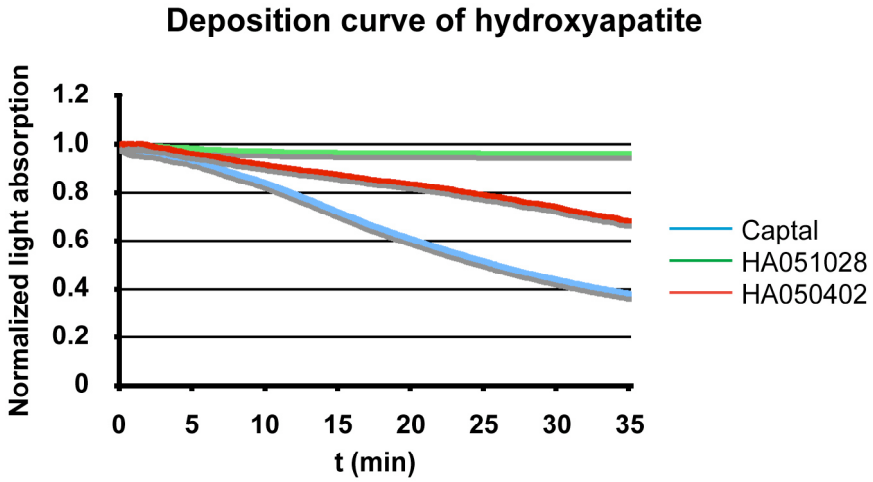

Figure 23: Deposition rates of commercial and tailor-made HA microparticles.
The deposition rate of commercial HA Captal® was compared to that of two tailor-made HA obtained in our laboratory. All the particles were meshed to use particles between 80 and 100 μm for this experiment and the XRD spectra of the HAs Captal®, HA051028 and HA050402 can be found in **Figure 22**. Deposition rates are obtained by plotting the normalized light absorption of 0.24 mg / ml of each HA in time; a lecture of absorption was obtained every 10 seconds during 35 minutes and referred to the initial absorption.

5.2.2. Surface coatings affect HA dispersibility

Charges inside biological scaffolds need to be as integrated and dispersed within the tissue as possible. We speculated that the coating of a biocompatible ceramic such as HA would produce a versatile material that would have better properties as biological charge. Thus, we have evaluated the coating of commercial HA or tailor-made HA with the biocompatible hydrophilic polymer polyacrylic acid. We also tried to use a polymerized organosilane as an interface polymer between HA and the polyacrylic acid to increase the quality of linkage between these two phases.

Coating of synthetic HA with polyacrylic acid was performed by cold plasma polymerization following the instructions of previous works [28, 136, 137]. Cold plasma was obtained inside a downstream plasma reactor developed in the group [28, 145] that is schematized in **Figure 29 A**. Briefly, this reactor consists on a cylindrical tube connected to a vacuum source that allows generating an atmosphere of monomer at a pressure that can be adjusted from 0.01 to 0.6 mbar. At these conditions, plasma can be obtained by inductive radiofrequency (RF) activated plasma and consequently monomers polymerize.

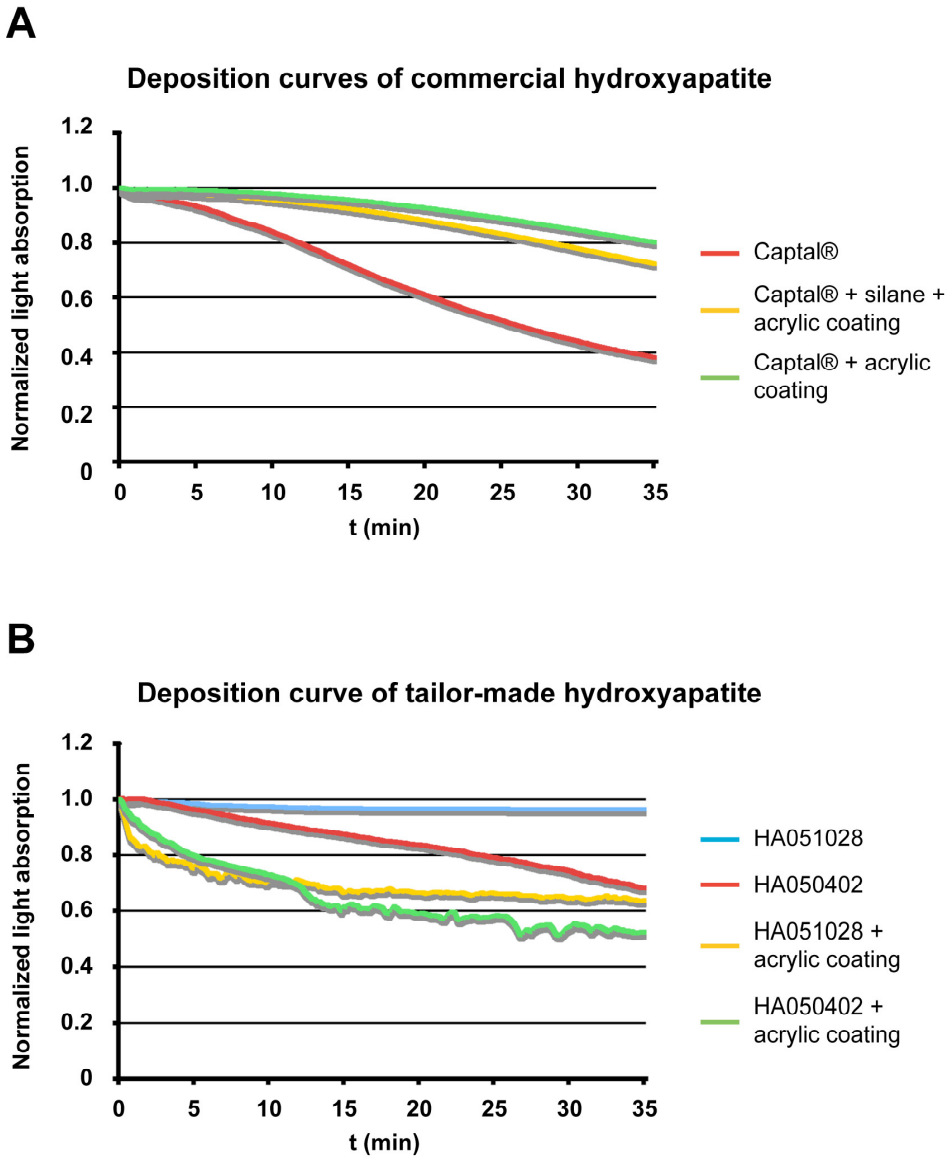

Figure 24: Deposition rates of coated HA microparticles.
(**A**) Captal® microparticles were coated first with a silane coating and then with an acrylic coating by plasma polymerization. The resulting particles were meshed to use particles between 80 and 100 μm for this experiment. Deposition rates of all these modified Captal® microparticles was compared to that of Captal® (**B**) Tailor-made HAs HA051028 and HA050402 were coated with acrylic acid by plasma polymerization and meshed to obtain particles between 80 and 100 μm. Deposition rates of these coated and uncoated microparticles were obtained. In both experiments, deposition rates are obtained by plotting the normalized light absorption of 0.24 mg / ml of each HA in time; a lecture of absorption was obtained every 10 seconds during 35 minutes and referred to the initial absorption. The XRD spectra of the HAs Captal®, HA051028 and HA050402 can be found in **Figure 22**.

Comparing the deposition rates of coated and raw tailor-made synthetic HA microparticles **(Figure 24 B**) we can infer that, surprisingly, uncoated HA microparticles are more dispersible than the polyacrylic coated ones. Solubility is usually related to the interaction's degree between a solid and the medium it is suspended in. Thus we had wrongly predicted that HA, a quite insoluble calcium phosphate would have lower dispersibility in water than HA coated with a well-known hydrophilic polymer such as polyacrylic acid. Results show that synthetic HA has very good, even excellent (HA051028) dispersibility compared to the coated HA (**Figure 24 B**). Nevertheless, these results are consistent with another study in the group that pointed out that synthetic HA had a very high capability of having Ca^{2+} exchange with soluble Ca^{2+} from a simulated body fluid [27, 28]. Furthermore, the synthetic HA we obtain, though insoluble, has a fairly high solubility in deionized water (results not shown).

We also tried to use the organosilane 3-aminopropylsilane (AMMO) as a sticking interface between HA and the organic polymer in case it conferred better properties to the resulting coated HA. The organosilane was fixed on Captal® by dip-coating and aged with a posterior thermal treatment. Captal® activated with AMMO was then submitted to plasma polymerization of polyacrylic acid under the same conditions explained above. The results show that there is no significant difference between the deposition kinetics of silane activated HA coated with polyacrylic acid compared to HA coated only with acrylic acid (**Figure 24 A**). More interestingly, both coated HA microparticles are more dispersible than the plain HA ones (**Figure 24 A**). This piece of data contrasts with the results obtained with our synthetic HA, where plain HA was more dispersible than the coated one. Nonetheless, this is not surprising because the physical properties of Captal® and synthetic HA are quite different. For instance, Captal® is less soluble in deionized water than synthetic HA is. XRD spectra from Captal® show that Captal® is much more crystalline than synthetic HA, probably due to a more intensive thermal treatment during the synthetic process. Its low solubility in water and strong crystalline structure indicate that Captal® is a quite apolar ceramic, which would be directly related to its poor dispersing capacity in a simulated body fluid. Therefore, the hydrophilic coatings tried in this experiment increase the dispersibility of Captal® microparticles.

5.2.3. Doehlert method to optimize grafting polymerization of PFM on 96-well plates

The fist step towards the production of PFM modified plates is to measure the efficiency of coating depending on the reaction conditions. To obtain a quantitative and trustable graph representing the efficiency of coating we used a Doehlert design to obtain a surface response relating the quantity of PFM attached on the surfaces to the concentration of PFM and time used in the coating reaction. These two parameters we studied refer to the reaction between PFM and the activated surface, where we evaluated the concentration of PFM in acetonitrile used in the reaction and the time the PFM was let react with the 96-well plate. The quantity of PFM fixed on the 96-well plates was measured by letting PFM react with glycine, which was then washed, hydrolyzed and analyzed by HPLC. Assuming that the quantity of glycine we

detected by HPLC in each of our experiments was equimolar to the quantity of PFM attached on the wells' surfaces, the quantity of glycine detected in the Chromatograms corresponds to the quantity of PFM attached on the wells. The quantity of glycine present in each well was obtained using artificially added alanine as internal standard for the normalization of the results. An example of the chromatograms that we obtained from the samples is represented in **Figure 25**.

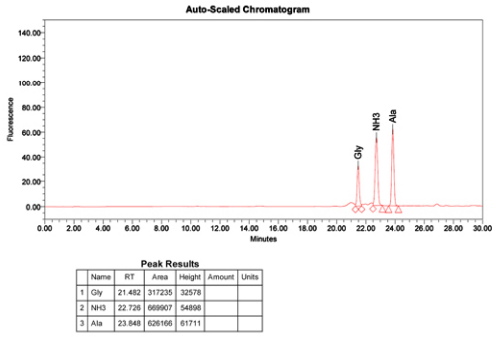

Figure 25: Chromatogram of glycine quantification by HPLC

The molecules of glycine quantified by HPLC correspond to the molecules of PFM attached to the surface of the wells. For plotting reasons, the quantity of PFM fixed on the plate surface was expressed as the % of surface occupied by the PFM molecule. This value was obtained by comparing the area of the well to the area occupied by the molecules of PFM fixed in the well. The minimum surface of a single molecule of PFM was obtained by theoretical calculus using Chemsketch© software and amounted for 34.96 A^2 so the total area occupied by PFM was obtained by the following calculus:

$$\%\ \text{Occupation}\ \ \text{PFM} = \frac{\text{molecules}\ \ \text{PFM} \cdot S_{PFM}\ (A^2)}{S_{well}\ (A^2)}$$

We converted the moles of PFM attached to each well into % of occupation of PFM on the well surface and then adjusted the predictive Doehlert function. The Doehlert design adjusts a second order function to relate the response, % of PFM in the well surface (Z), with the two factors studied, concentration of PFM (X) and time of reaction between activated surface and PFM (Y). The second order function adjusted is the following:

$$Z = a + b \cdot X + c \cdot Y + d \cdot X \cdot Y + e \cdot X^2 + f \cdot Y^2$$

Were the adjusted constants were found to be:

$$a = -0.359759051550043$$
$$b = 0.00763894988049824$$
$$c = 0.0211311355613675$$
$$d = -3.51429394692153 \cdot 10^{-5}$$
$$e = -1.80937597813868 \cdot 10^{-5}$$
$$f = -1.69404965113536 \cdot 10^{-4}$$

We evaluated the significance of this regression by analysis of the variance. The analysis of variance showed that the depicted surface represents the experimental points with a signification (p) below 0.05 (**Figure 26**) and the residual error can be considered as pure error with $p<0.05$.

Source of variation	Degrees of Freedom	Sum of Squares	Mean Square	F ratio
Regression	5	1.969	0.393	15.7[a]
Residuals	8	0.200	0.025	
Lack of fit	1	0.077	0.077	4.42[b]
Pure error	7	0.123	0.018	
Total	13	2.169		

[a] $15.7 > F_{crit} = 3.69$ (with 5 and 8 degrees of freedom and $\alpha = 0.05$)
[b] $4.42 < F_{crit} = 5.59$ (with 1 and 7 degrees of freedom and $\alpha = 0.05$)
Figure 26: Summary of the ANOVA for the response % of PFM occupation (Z)

The adjusted function can be plotted and generates a surface response that shows the predicted Z (% of PFM occupation) for each defined X (concentration of PFM) and Y (time of reaction). The Doehlert surface obtained (**Figure 27**) depicts a dark red area where the most efficient coating with PFM is obtained. This area represents: I) a concentration of PFM in acetonitrile range between 175 and 400 mM; II) a time of reaction between the PFM solution and the plasma-activated well plate between 55 and 80 or more minutes. In this experimental domain, coating with PFM achieves values from 140 % up to 178 %. These values are obviously overdimensioned probably due to both the coating of the lateral walls, which would increase the effective modified area, and the fact that this % of PFM occupation is based on a theoretical value for the molecular surface of PFM. Moreover, we have considered that the poly(PFM) gives rise to a flat thin film after grafting, which is not very likely, because grafting polymerization has few initiation spots (corresponding to ionized dots on the plates' surface) where the polymer grows. In these conditions, the polymer probably resembles more the shape of a dunned desert than the shape of a flat lake. The modification of the effective surface area could also explain the abnormally high values for the percentage of occupation of the plates' wells.

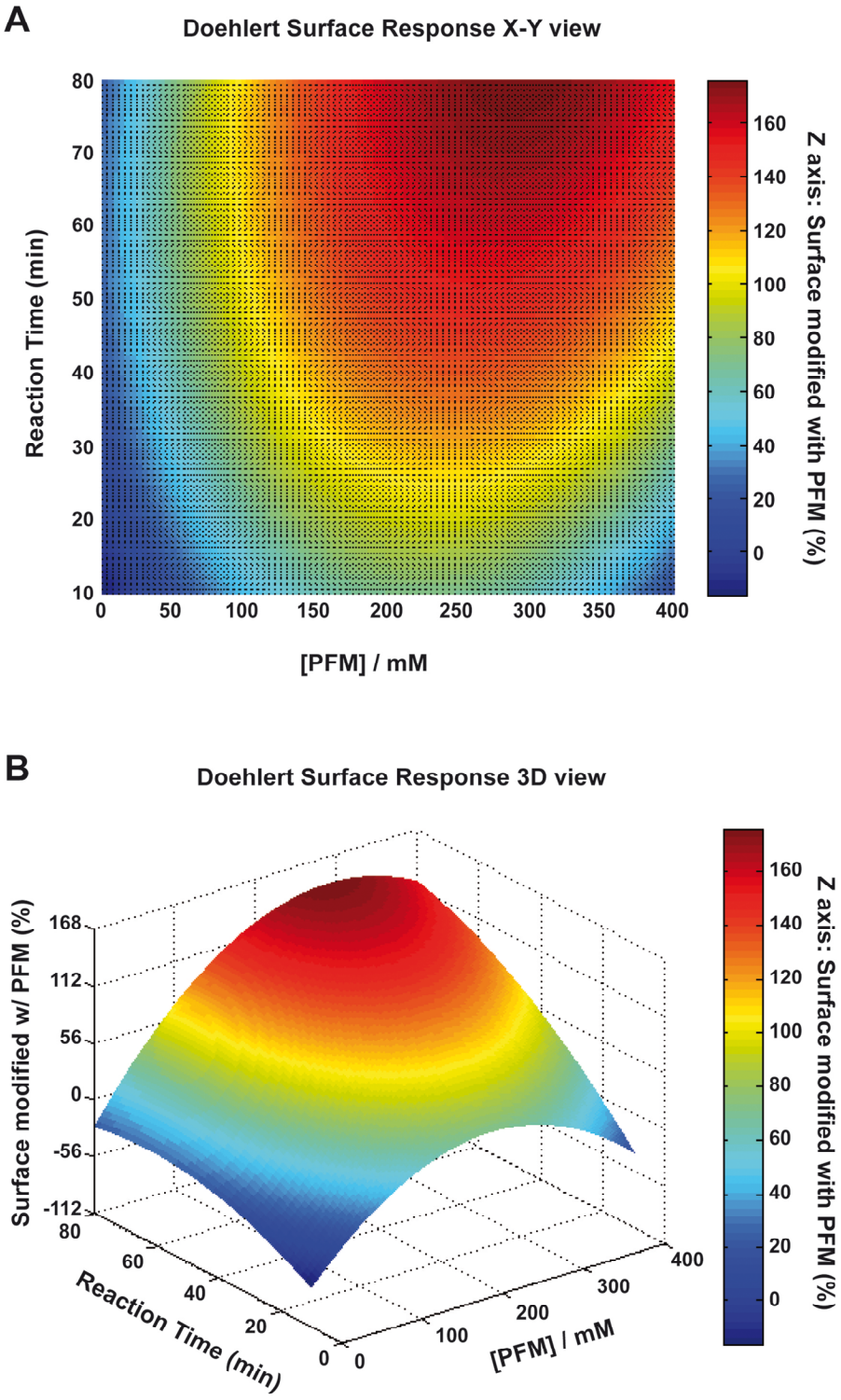

Figure 27: Doehlert Surface representation of the predicted % of PFM occupation. The adjusted Doehlert function has been plotted using MATLAB© software. (**A**) 2D (X-Y view) of the Doehlert surface. (**B**) 3D view of the Doehlert surface.

A side effect of the reaction between PFM dissolved in acetonitrile and the 96-well plate was the outcome of fractures within the whole plate. After reaction with PFM, a collection of organized and oriented fractures appeared in the wells (**Figure 28**). These fractures did not brake nor compromise the wells' performance so we could follow with our experiments and attach glycine to the wells. Nevertheless, the fractures seriously damaged the optical properties of the plate and were not favorable for cell culture (results not shown). Thus, future applications should consider the use of another solvent to dilute PFM that is not harmful to the well plate (water, acetonitrile+water…).

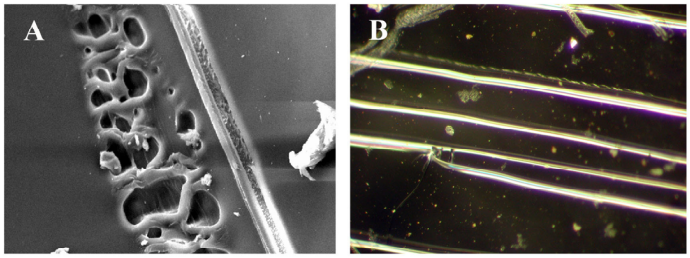

Figure 28: Visual aspect of PFM coating of 96-well plates.
(**A**) 750 X SEM image of a coated well acquired with a JSM 5400 Scanning Electron Microscope. The image shows a fracture of the plate on the right and a collection of holes that correspond to discontinuities of the PFM coating on the plate. (**B**) 5 X image of the fractures that appear in the 96-well plates after the coating with PFM. The image was acquired with an Olympus stereo microscope SZ-PT.

5.3. *Discussion*

There has been extensive work done in developing charges to be applied in constructs for biomedical applications [146]. Hydroxyapatite (HA) is the naturally occurring mineral charge present in calcified cartilage and bone so many of the approaches to produce tissue engineered materials for bone and cartilage regeneration have used HA itself or some type of modified HA [99, 146]. We tried to contribute to this field by developing surface modified HAs to improve their interaction with their biological surrounding. More specifically, we wanted to label HA with RAD16-I and mix these particles with RAD16-I hydrogels to produce synthetic constructs with the ability to reinforce biomimetic or biological scaffolds for tissue engineering. With this in mind, we first performed a proof of concept of our ability to coat HAs with organic polymers and sideways to obtain a more easily dispersible type of HA. We have also compared the performance of commercial and tailor-made synthetic HA.

Regarding our results, the first remarkable aspect is that commercial and synthetic HAs have different chemical behaviors. Although we haven't properly tested properties such as the biocompatibility, osteoconductivity or Ca^{2+} release of these HAs, they are very likely to have different characteristics and overall performance *in vivo*. On the one hand, some biomedical applications such as coating of implants with HA to increase their osteoconductivity, require highly crystalline and insoluble HA. For this purpose, Captal® would be the first choice as it is less soluble and interactive with body fluids than is synthetic HA (**Figure 23**) [147, 148]. On the other hand, some other applications require the use of HA capable of releasing Ca^{2+} to their surroundings, which is the case of calcified cartilage constructs for applications in articular joints. For this application, it is important that HA has a good calcium release and that HA is highly dispersible for it will be suspended in a hydrogel-like matrix such as RAD16-I, as in our particular approach, collagen type I or Matrigel®. In this case, our synthetic tailor-made HA would be the first choice to include as charge in engineered tissues for cartilage repair because it best meets the two most important requirements, calcium release and dispersibility, that any HA needs to render a functional cartilage joint substitute.

A second aspect of interest is the fact that coating of HA renders particles of different nature depending on the source of HA used to produce the bulk microparticles. Captal® is a very insoluble type of HA and the coating of Captal® with polyacrylic acid gives rise to more dispersible and thus more hydrophilic particles. Instead, tailor-made synthetic HA microparticles present a high hydrophilicity on their own and the coating of these particles, even with a hydrophilic polymer, renders a more hydrophobic product that is less dispersible than the original HA microparticles. The different response of the two types of HA to polyacrylic coating strengthens the fact that commercial and synthetic HA powders will behave differently. Moreover, this different response to organic coatings allows us to better control the final properties of HA, as we can produce HA of a wide range of dispersibilities,

thus enabling us to tune our HAs for optimal performance in cartilage joint engineered constructs.

We hypothesized that a strong link between HA and the polyacrylic coating we applied would increase the effect of the coating as it would avoid spontaneous degradation of the polymer. Organosilane coatings have long been used with the purpose of bridging inorganic and organic phases [128, 149-152] so we decided to coat HA first with a common organosilane (AMMO) that is routinely used to coat glass slides in microscopy. These silanized microparticles were coated with polyacrylic acid and compared with HA microparticles coated only with polyacrylic acid. Considering that dispersibility of the microparticles reflects their hydrophilicity, the presence of a silane bridge between HA and the organic coating has no significant benefit to the hydrophilicity of the particles (**Figure 24**). Although more than probable, it remains to be proved that this organosilane bridge strengthens the link between HA and any organic coating, which would also presumably increase the durability of the coating. Moreover, silane-treated HA particles are less soluble than plain HA particles [128] so the durability of silanized microparticles would also increase from this point of view. Altogether, silane coatings can be used as a mean to tune the durability of coatings on HA microparticles to best match any particular biomedical application.

After having obtained biomimetic inorganic charges for use in cartilage repair, we pursued to generate a platform to test growth factors, peptides or whatsoever that would allow us to find supplements which if included in a particular construct for cartilage repair would optimize its performance. With this in mind, we used organic coatings to functionalize 96-well plates' surfaces in order to obtain versatile platforms with wells containing a reactive group that would allow performing biochemical or biological combinatorial assays. We chose PFM as reactive group following a previous research line [136, 137] and used a Doehlert matrix design to optimize the coating of polystyrene plates with PFM by grafting polymerization.

First thing to comment is that in order to obtain this Doehlert surface response we have assumed that the quantity of glycine fixed on the PFM-modified 96-well plates, which was measured by HPLC, represents the quantity of PFM fixed in the surface. This assumption is not entirely correct because the yield of the reaction between glycine and PFM affects the overall quantity of PFM we obtain by this quantification procedure. However, our goal is to compare different operating conditions among them and, considering that the yield of the reaction between PFM and glycine will be equal in all wells, the aspect of the surface will not be affected by this assumption. Only the absolute values represented in the curve can be underestimated, meaning that the Z values in the curve might all of them be higher than they really are, but without changing the shape of the surface response.

The Doehlert regression rendered an area depicted in dark red in **Figure 27** that represents the best working conditions for our experimental set-up: a concentration of PFM between 155 and 400 mM and a time of reaction of 55 to 80 min. This multi-factorial second order function

has a maximum than can be calculated with the derivates in X and Y of the function Z and the eigenvalues of its Hessian matrix. The maximum Z (% Occupation of PFM) is obtained when X ([PFM]) = 302 mM and Y (T reaction) = 94 min. Nevertheless, this maximum doesn't need to be exact, a new Doehlert design on the same experimental map centered on this calculated maximum [(X,Y) = (302,94)] would ensure that these conditions are those giving rise to surfaces with higher PFM coating rate.

As previously said, % of occupation of PFM gives values over 100 % because the walls of the well might also be coated and reactive, the calculated value for the molecular surface of PFM might be inaccurate and the topography of the coating is probably not flat, increasing the total effective surface of the wells. However, the results still allow us to identify the experimental domain that renders better PFM coatings of 96-well plates, confirming that the Doehlert design is a useful tool to extract the maximum information with the minimum time and costs. This information, stored in the surface response depicted in **Figure 27**, will help future works in choosing the working conditions that will be most appropriate for a particular application. Not necessarily a more efficient coating with PFM will ensure a better platform for combinatorial assays. For instance, a platform for the study of growth factors, that are usually big proteins, might be better with fewer amounts of PFM molecules on the surface because a high concentration of PFM on the surface might favor the presence of multiple binding sites between proteins and the surface. Proteins tightly bound would have fewer degrees of freedom to adjust their quaternary structure to fit their receptors so the platform might give inaccurate information about activity of these proteins. The surface response we have obtained in this thesis will help us in navigating through the experimental conditions to find an appropriate PFM coating for a particular combinatorial application.

A problem that appeared during the grafting polymerization of PFM on polystyrene plates was that the polystyrene developed fractures when in contact to acetonitrile (**Figure 28**). This is probably due to inherent tensions that remain in the plates after the extrusion process by which they are manufactured. These tensions are fragile spots that when in contact to a solvent that partially dissolves the polystyrene, develop into small fractures, as reported above. Therefore, the grafting process we propose needs to be changed to avoid the use of a solvent in which polystyrene is partially soluble. Work from Francesch et al. [136] has shown that the reactivity of PFM with water is very slow compared to the reactivity of this group with amines. This opens the possibility to use the same experimental setting changing only the reaction solvent for water. Thus, the surface response obtained in this experiment will guide future work in the group in order to establish a robust process for the production of PFM modified plates.

According to the Doehlert regression, a good compromise among time, costs and coating efficiency would be achieved by the following method: I) the 96-well plate is activated by Ar/O_2 continuous plasma during 60 min at 100 W. II) The wells of the activated plate are

soaked with a solution of 200 mM PFM during 55-75 min at room temperature. III) The solution is removed, the wells are washed and the plate is stored in inert atmosphere for long storage. The solvent still remains to be adjusted, although water seems to be the best choice.

In summary, we have developed different types of hydroxyapatite microparticles that have different dispersibility depending on the origin of the HA and the presence of an organic coating. We have also given more evidence that commercial and synthetic HA have different behaviors, this confirming previous research [29]. Finally, we have developed a procedure to produce surface coated 96-well plates by using a Doehlert experimental design. The results rendered an experimental domain that efficiently coats the plate. Nevertheless, the process compromises the structure of the plates so we need to still work on some parameters such as the solvent of the reaction with PFM.

5.4. *Materials and methods*

5.4.1. Coating of particles

5.4.1.1. Synthesis of hydroxyapatite (HA)

Hydroxiapatite microparticles were obtained by the sol-gel synthetic method, as previously described [26]. Briefly, calcium nitrate (Sigma, C1396) and partially hydrolyzed triethylphosphite (Sigma, 538728) were mixed in an aqueous solution to obtain a Ca/P ratio of 1.67, which is that of hydoxyapatite crystals. The solution remained 48 h at room temperature without agitation to let crystal nuclei form. After that, the solution was heated at 60 ºC until a sol formed and then it was jellified at room temperature. The gel was washed to get rid of nitrates and carbonates and then heated at 400 ºC during 3.5 h. The resulting powder was grinded and sifted to obtain microparticles of a regular size, between 80 and 100 μm. XRD was used to evaluated purity and crystallinity of the synthesized hydroxyapatite (**Figure 22**). Occasionally, some HAs such as HA050422 (**Figure 22**) were heated at 600 ºC in the final thermal treatment of the sol-gel protocol and the powders presented a higher degree of crystallinity.

5.4.1.2. Coating of HA with 3-Aminopropyltrimethoxysilane (AMMO)

Commercial HA microparticles (Captal®) or synthetized HA microparticles were sifted in order to obtain microparticles with a particle size between 80 and 100 μm. These microparticles were suspended in a water-free Acetonitrile solution of AMMO ($25\%_{(v/v)}$) and stirred during 2.5 h. Hydroxyapatite microparticles were filtered, dried at room temperature and cured at 100ºC for 24 h. A slightly yellowish glassy powder was obtained. This powder was again sifted to obtain particles between 80 and 100 μm.

5.4.1.3. Coating of HA microparticles by Plasma polymerization

HA microparticles were placed in a platform in a downstream plasma reactor as showed in the following scheme (**Figure 29 A**). This reactor was previously used in our group to coat different types of powders [27, 28, 145, 153]. The hydroxyapatite powder was treated with downstream plasma at a pressure of 0.2 to 0.4 mbar of acrylic acid during 60 minutes. 60 W of continuous power was supplied to the reactor to ionize the monomer.