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Review

A Review on Ionic Substitutions in Hydroxyapatite Thin Films: Towards Complete Biomimetism

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Abstract: Plasma sprayed coatings composed of stoichiometric hydroxyapatite have been extensively used to improve integration of metallic implants in the host bone, as hydroxyapatite (HA) is normally regarded as similar to the mineralized phase of bone. However, these coatings exhibited several drawbacks that limited their success. On the one hand biological apatite is a carbonated-HA, containing significant amounts of foreign ions, having low crystallinity and a small crystals size. This means that it differs from stoichiometric HA in terms of composition, stoichiometry, crystallinity degree, crystal size/morphology and, as a direct consequence, solubility, and ions release in the peri-implant environment. On the other hand, thick plasma sprayed coatings can undergo cracking and delamination and are scarcely uniform. For these reasons, research is pushing into two directions: (i) Increasing the similarity of apatite coatings to real bone, and (ii) exploring deposition by alternative plasma assisted techniques, allowing to achieve thin films, and having superior adhesion and a better control over the coating composition. In this article, we review the latest advances in the field of plasma-assisted deposition of ion-substituted hydroxyapatite thin films, highlighting the state of the art, the limitations, potentialities, open challenges, and the future scenarios for their application.

Keywords: calcium phosphates; ion-substituted apatites; bone regeneration; plasma-assisted deposition; solubility; crystallinity; composition

1. Introduction

Orthopedic implants are still burdened by high failure/revision rates, posing relevant economical and societal challenges [1–13].

To avoid failure, implants must integrate in the host site, which requires the capability to trigger host bone cells response without eliciting any immune reaction [14,15]. However, implants also need to burden physiological loads, which can be high and multiaxial, and must not be fragile, hence why metallic implants are normally used, that are bio-inert. A common route to overcome bio-inert behavior of metallic implants and boost integration is to coat their surface with layers having osseointegrative capabilities, so as to render the implant bio-active [16,17]. These coatings are traditionally manufactured by plasma spraying, and have quite significant thickness (comprised between ~30 and 200 μm) [17].

According to the "biomimetic principle", the biological behavior of a device for bone replacement can be boosted by using a biomaterial as similar as possible to real bone in terms of its composition, crystallinity, lattice dimensions, and Ca/P ratio [18]. This, in the field of bone regeneration, has pushed towards an extensive use of hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$, HA), as a bone substitute, in view of its similarity to the inorganic phase of mineralized tissues of vertebrates [16,17,19].

However, clinical results obtained by these traditional coatings are not as promising as expected, and generally not superior to those obtained by bare titanium. This can be ascribed to two main reasons:

(i) Despite the assumed similarity between biological apatite and stoichiometric apatite having been referred to for decades, they exhibit significant differences, from several points of view. Firstly, in terms of composition, bone apatite is a multi-substituted carbonated HA [18,20,21]. Each substitution can influence the characteristics of the lattice, thus having an impact on its stability, crystallinity degree, crystal size and morphology, all influencing its solubility and, ultimately, ion release in the biological medium [18,20,21]. In addition, several of these ions can have a significant biological role, directly influencing host cells response and/or exerting a therapeutic role, generally in a dose-dependent manner, hence their presence and amount in the peri-implant environment is significant [18,21].

(ii) Due to relatively high thickness, plasma sprayed coatings have a high tendency to cracking and delamination [16,22,23]. In addition, coating manufactured by PS exhibit scarce uniformity in terms of thickness, phase composition and microstructure [16,22,23]. These two aspects have been hampering the performance of these materials.

For this reason, in the last years, research has been pushing into two directions, to overcome these issues:

(i) Ion-substituted apatites are being investigated, instead of stoichiometric hydroxyapatite.

(ii) Novel plasma-assisted techniques have been emerging, allowing to deposit thin films, with superior adhesion to the substrate and lack of tendency to crack.

In addition, an even more recent route is to deposit coatings directly from bone apatite precursors, which is made possible by plasma-assisted techniques.

Here, we review the literature regarding deposition of ion-substituted apatites by novel thin film plasma-assisted techniques, with special attention to deposition from biological precursors, to highlight the potential, issues and challenges connected to this approach. The focus is on coatings aiming at promoting osseointegration, by a composition as similar as possible to the real bone, hence the Review will be centered on substitution by ions present in the bone. However, because ion-substituted apatites coatings are getting increasing attention for several applications and especially for infections, literature regarding silver-doped hydroxyapatite will be briefly discussed in Section 6, which discusses future perspectives on this topic.

2. Plasma-Assisted Methods for Calcium Phosphate (CaP) Deposition

Plasma-assisted techniques offer some advantaged compared to wet methods and to traditional plasma spraying. Compared to wet synthesis, they allow for much faster coating of the surface thanks to generally high deposition rate, which is an important advantage from an industrial point of view [21]. In addition, they are industrially scalable, which is also boosting their application. Compared to plasma spraying, they allow for an easier transfer of the target composition, a better uniformity in terms of morphology and composition, a lower porosity and a lower tendency to cracking and delamination [16,22].

Among plasma-assisted techniques, the most used in the biomedical field, for manufacturing thin films for bone implants are radio-frequency magnetron sputtering (RF-MS) and pulsed laser deposition (PLD). Matrix-assisted pulsed laser evaporation (MAPLE) and pulsed electron deposition (PED) are emerging techniques, allowing a fine control over film stoichiometry and composition and are gaining increasing interest, hence they will be included in the review.

In magnetron sputtering (MS), deposition takes place starting from the ionization in vacuum of a target material by means of negatively charged magnets [17]. Although two different modes are available, direct current (DC) and radio-frequency (RF); CaP coatings (and, more in general,

non-conductive materials), can be deposited only by the latter [17]. MS allows an easy deposition of multi component coatings, by deposition from multi-component ceramic targets or simultaneous deposition from different targets [24]. Coatings can be very thin (average thickness being ~40 nm–3.5 μm) and can effectively cover surface irregularities, while maintaining a uniform thickness. In addition, they are dense and pore-free [16]. As for all plasma assisted techniques, substrate temperature and deposition time are critical in determining the characteristics of the produced films, but in the case of MS those also have a key role on composition [16]. Films can be deposited at room temperature, which allows coating of heat-sensitive substrates; however, scarce adhesion has been reported for deposition at room temperature, in absence of substrate preparation and/or post deposition treatments. As for all plasma assisted techniques, as-deposited films are amorphous, but both deposition at high temperatures or post deposition annealing are effective strategies to obtain the desired crystallinity degree. Post deposition annealing also allows to tailor surface morphology and roughness to a certain extent. Deposited coatings are generally highly adhesive (bonding strength to the substrate >30 MPa) and exhibit suitable bioactivity. One of the main limitations of this technique is that the coatings composition might differ from that of the deposition target material, also depending on sputtering system and parameters. This, for calcium phosphates, can be very relevant, as significantly different properties can be obtained for slight variations in composition, Ca/P ratio and amount of ionic substitutions. In particular, the Ca/P ratio of the films might vary in a very wide range (1.6 up to 2.6 or even higher) and is normally higher than that of the HA target, due to preferential sputtering of calcium and/or to evaporation of P_2O_5 [16,24].

In PLD, the target to be deposited is ablated by a focused, high power, pulsed laser beam. As a consequence, a plasma plume forms that is accelerated towards the substrate where deposition occurs. Coatings obtained by PLD can be very thin and much below 1 μm. The characteristics of the deposited films are strongly dependent on deposition parameters that determine their morphology, composition and crystallinity. As for MS, as-deposited films are amorphous, but higher crystallinity can be achieved depending on deposition temperature and possible post-deposition heating treatments. However, because deposition can occur at room temperature, coating of heat sensitive substrates is allowed. Substrate temperature and post treatment annealing also have an impact on the final characteristics of the coatings and in particular on the composition and stoichiometry of the coatings. Despite this, PLD allows for a fine transfer of the target composition. This precise control over stoichiometry makes it possible to deposit multicomponent coatings from one same target [16,17,25,26]. Finally, PLD coatings generally exhibit high adhesion to the substrate, good biocompatibility and bioactivity.

When delicate organic or biological matter or thermally unstable phases needs to be deposited, a modification of PLD can be used, namely Matrix-assisted pulsed laser evaporation (MAPLE). MAPLE prevents the laser source from damaging the target, that is a frozen mixture of the "active" biomaterial (up to 5 wt.%) in volatile solvent, gradually removed by a vacuum system as the deposition proceeds [17,27,28]. The solid target is kept frozen during laser irradiation, so as to prevent evaporation; this is achieved by a cooling system inside the chamber and instant-freezing of the targets by immersion in liquid nitrogen. The solvent is chosen to absorb the laser wavelength, that is also tailored to prevent damage of the target, thus avoiding interactions between the laser and the material, allowing the release of the undamaged delicate active matter, that is deposited on the substrate.

More recently, pulsed electron deposition has been proposed as an alternative to PLD also for applications in the biomedical field [29–31]. In PED the target material if ablated by a high energy pulsed electron beam instead of a laser source; a plasma plume is formed at each pulse and the target material is ionized and deposited onto the substrate [32]. Due to the lower cost of the ablation source, PED is more competitive from an economical point of view, compared to PLD. As in the case of PLD, films deposited by PED are composed by globular aggregates and exhibit high roughness, which makes it suitable for promoting host cells behavior [33]. Films obtained are amorphous, but post deposition annealing can be performed to obtain the desired crystallinity degree [34,35]. Films obtained are highly

adhesive, adhesion also being influenced by post deposition annealing [36]. Mechanical properties achieved are comparable to those obtained by plasma spraying [36]. Finally, good biocompatibility and bioactivity have been reported [33].

3. The Role of Ionic Substitutions in Hydroxyapatite

The main ionic substitutions present in bone are reported in Table 1. The most diffused substitution is CO_3^{2-} for OH^- (A-type substitution) or for PO_4^{3-} (B-type substitution), followed by and Cl^- and F^- for OH^- [19]. In addition, magnesium, strontium, zinc, and manganese substitutions for calcium, and silicates for phosphates are also present in bone [20,21,37,38]. The amount of these substitutions can significantly vary between different tissues (i.e., bone, dentine, enamel), animal species, types of bone, anatomic sites, etc. [21]. In addition, significant differences can be assessed depending on the patient age, sex, and pathologies.

Ionic substitutions are relevant, as each of them have an influence on HA crystal lattice and hence on its solubility. In addition, some of them have significant biological impact and can have therapeutic effects [19,21]. Those have been largely investigated to obtain substitute HAs for bone regeneration and are their role is reviewed below.

Carbonates cause a decrease in *a*-axis and decrease in *c*-axis of HA, this renders the structure is less crystalline and, as a consequence, more soluble and more bioactive [19,21,39]. Magnesium, the main substitution for calcium in HA, causes a decrease in the *c*-axis of the lattice, acts as an inhibitor of HA nucleation and crystallization and destabilizes its structure. Because Mg-HA is less crystalline than stoichiometric HA, it also has higher solubility [19,21,40]. In addition, Mg is an essential element for living organisms, as it is a key factor in the activity of several enzymes [40]. In bone, Mg influences metabolic activity and growth, by acting on osteoblasts and osteoclasts. As a consequence, scarce concentrations of magnesium can cause osteopenia or fragility [40]. Fluorine substitution for OH^- causes a decrease in the *a*-axis and in the c-axis, the latter being lower by an order of magnitude compared to the first. As a consequence, the lattice gains a strong symmetry, that results in an increased stability and a lower solubility [21]. The effect on bone cells, instead, is controversial, as F^- itself can boost cells attachment, but the lower solubility causes a reduced availability of Ca^{2+} ions decreasing cells proliferation. Lastly, fluorine is expected to be an effective anti-osteoporotic agent [21,41]. Strontium can be incorporated in bone by both ion exchange and ionic substitution [19]. The effect of substitution of Sr for Ca is dose-dependent: High concentrations of Sr increase crystals dimensions and crystallinity, while low concentrations cause an opposite effect (decrease in crystallinity and crystalline length), and a distortion of the lattice, both leading to a significant increase in solubility [19]. Interestingly, solubility can be further increased by Sr substitution in carbonated hydroxyapatite (CHA), instead of HA. From a biological point of view, Sr increases the mechanical strength of bone [21] and has an anti-osteoporotic effect [19–21]. In fact, strontium ranelate, a strontium-based drug used against osteoporosis, can boost osteoblast activity while inhibiting osteoclasts [20,21,24,37,38,42,43]. Sr capability to improve bone regeneration has also been demonstrated by tests in vivo [17,20,37,38,42]. As for the effect on crystallinity and solubility, also in vitro and in vivo behavior of Sr is dose-dependent. Silicon is normally incorporated in biogenic HA because of SiO_4^{4-} substitution for phosphate groups [19]. This substitution increases HA solubility, also because formation of amorphous phosphate phases is promoted, the apatite microstructure becomes finer and, finally, silicates form defects at grain boundaries [19]. Even at concentrations of about 1 wt.%, silicon can exert significant biological impact and it is reported to enhance in vivo bioactivity and bone regeneration [44]. Zinc is a trace element in bone [19,21], that can substitute for calcium, thus acting as a crystallinity inhibitor. Despite its low concentration, it has a key biological role, as it can act as a co-factor for several enzymes, can impact on nucleic acid metabolism and promote bone formation and regeneration by favoring osteoclasts activity and decreasing that of osteoblasts [19,21]. Manganese is also a trace element, acting as a crystallinity inhibitor [19]. Depending on the concentration, it can alter HA morphology to a different extent, which

has an impact on the apatite bioactivity [19]. Mn has a role in bone remodeling and the influence of Mn-doped carbonated hydroxyapatite on osteoblasts activity has been demonstrated [19,29,45].

4. Deposition of Ion-Substituted Apatites by Plasma-Assisted Techniques

A scheme of literature about plasma assisted deposition of ion-doped apatites in reported in Table 1.

Table 1. Available literature on ion-substituted coatings realized by thin film deposition techniques. F: Only data relative to coating fabrication and characterization available; V: Data from biological in vitro test available; VV: Data from biological in vivo test available.

Deposition Technique	Carbonate			Mg			F			Sr		
	F	V	VV	F	V	VV	F	V	VV	F	V	VV
MS		[46]			[47]		[48]			[1,38]	[43]	
PLD	[39,49]		[50]	[51]	[40]	[52]				[42]	[19]	
MAPLE					[27]						[27,53]	
PED										[36]		
Deposition Technique	**Si**			**Zn**			**Mn**			**Biogenic Sources**		
	F	**V**	**VV**	**F**	**V**	**VV**	**F**	**V**	**VV**	**F**	**V**	**VV**
MS	[54,55]	[56,57]	[56]		[47]							
PLD	[53]	[58]					[49]	[45]	[50]	[26,29]	[27,28,33]	[35]
MAPLE												
PED										[34]	[33]	

4.1. Deposition by Magnetron Sputtering

Deposition of carbonated hydroxyapatite (CHA), magnesium-doped hydroxyapatite (Mg-HA), fluorine-doped apatite (FA), strontium-doped apatite (Sr-HA), silicon doped-apatite (Si-HA), and zinc-doped apatite (Zn-HA) has been reported by magnetron sputtering [39,46].

Regarding carbonated hydroxyapatite, high adhesion (measured by pull-out test) has been obtained for different substrates, further increased when co-sputtering of titanium is performed [46]. Quite fine control over stoichiometry of the films has been achieved (Ca/P 1.80) [46].

Sputtered CHAs are biocompatible, as no toxicity is registered towards host cells. Further, suitable adhesion, viability and proliferation of osteoblasts have been reported. In vitro tests showed mineralization after 21 days immersion in simulated body fluid (SBF), and human bone marrow derived MSCs differentiation in human osteoblasts-like cells (hOBs).

Mg-HA coatings have been deposited by targets with variable magnesium content. In vitro tests indicated high adhesion (by pull-out test) and viability of osteoblasts, though not superior to that obtained on pure HA. The authors ascribed this behavior to amorphous nature of both coating, leading to too high a dissolution, making it impossible to discriminate between the effects of the two.

Fluorine-substituted apatite, deposited on Ti and Ti/Si by right-angle MS, indicated a general decrease in HA grain size, independently from the substrate. Crystallinity of the coatings depends on deposition time, as short sputtering times (i.e., 5 min) led to disordered nanocrystalline phases, while longer times allowed for an increase in crystallinity [47]. However, either pre-heating or post treatment annealing (at temperatures up to 300 °C) allowed for significant increases in crystallinity of coatings deposited at room temperature. In particular, by pre-heating, suitable crystallinity can be obtained for temperatures lower than those required for post-deposition annealing [47].

Sr-HA has been deposited both by Sr-HA targets with variable Sr/(Sr + Ca) ratio and by co-deposition from different targets. Deposition on titanium alloys and silicon has been reported [1,24,38,43]. Interestingly, the content of Sr was found to influence sputtering rate.

Scarcely crystalline coatings were obtained, where successful incorporation of Sr in the apatite lattice was achieved [43]. Upon annealing, crystallinity of the films increases and morphology and adhesion experience modifications, as do the (Sr + Ca)/P ratio and the presence of by products (CaO and $Sr(OH)_2$). Coatings obtained are calcium deficient, as a Ca/P ratio of 1.18 was reported. A (Ca + Sr)/P ratio of 1.26 was found, that also is modified by annealing, that increases Sr content with respect to calcium. The (Ca + Sr)/P ratio is lower than that of the target, due to a preferential sputtering of Ca compared to Sr, deriving from different atomic weight. Deposition from multiple targets results in a generally higher ratio [1,38,43]. Remarkable adhesive strength to titanium has been reported—higher than that of stoichiometric HA [24].

As expected, Sr does effectively increase osteoblasts activity and decrease that of osteoclasts, which is clearly indicated by the fact that proliferation and differentiation is higher compared to pure HA [43].

Sputtered silicon-doped apatite (Si-HA) was deposited by both silicate-substituted HA targets and by co-sputtering of Si and HA targets, as seen for Sr. Different substitutions levels were obtained (i.e., $Ca_{10}(PO_4)_{6-x}(SiO_4)_x(OH)_{2-x}$, x = 0, 0.25, 0.33, 0.5) [48]. Silicates can be incorporated in significant amounts, as they can prevent incorporation of carbonates [3].

Even though all deposited coatings are amorphous, crystallinity degree depends on the amount of Si (crystallinity decreases for increasing Si content) [49]. Post deposition annealing does not influence the Si content in apatite, so that the desired crystallinity can be obtained without significantly modifying the coating composition. Deposition at high temperature (with progressive increase of temperature from room temperature up to 200 °C) has also been reported, causing the formation of a nanocrystalline coating, with some amorphous areas [48]. In our opinion, these latter results are significantly dependent on the annealing conditions chosen, and higher crystallinity could be achieved for higher deposition temperature, as reported elsewhere for other substituted apatites.

Despite the expected lattice distortion caused by incorporation of silicates in the lattice [49], no significant differences are detected between HA and Si-HA by X-rays diffraction. In addition to the relatively low amount of Si, this is ascribed by the authors to the proximity of silicon and phosphorous in the periodic table. Presence of secondary Si and CaO phases has been reported [50]. Despite a uniformity in the elemental composition within one coating [3,51], both stoichiometric and calcium deficient coatings have been produced [50,51], indicating that, on one hand, silicon does not tend to accumulate in specific areas, as seen, for example, in the case of magnesium. However, in our opinion, this also evidences a non-perfect and fully reproducible transfer of the target stoichiometry.

Silicates addition also have a dose-dependent impact on the mechanical properties (by nanoindentation) of the coating, as a general decrease in hardness and elastic modulus is registered and failure due to scarce adhesion (measured by scratch test) is reported for high Si concentrations [49]. In vitro, increased dissolution rate is found compared to HA [51], possibly to be ascribed to the decrease in OH- content due to increasing silicates substitution [52], that results in a decrease in HA grain size [49].

Dissolution in SBF was found to be significantly dependent on post deposition annealing [50], which is expected, as solubility strongly depends on crystallinity degree. Nevertheless, suitable mineralization and good activity towards bone cells were found for both annealed and non-annealed coatings [50]. Although the mechanism of interaction of Si with bone cells is yet to be fully understood, Si-Ha coatings were found to boost attachment, proliferation and differentiation of osteoblasts with respect to HA and uncoated implants and to favor the formation of an extracellular matrix [3,51,53]. In vivo tests on mice are reported, indicating that Si-HA coatings are osteoinductive, while stoichiometric HA coatings are not. Both coatings, however, are biocompatible [51].

Zn substituted HA has also been deposited by RAMS, but literature is very scarce in this regard, and no deposition is reported by any other plasma-assisted technique, to the authors' best knowledge. In vitro tests on Zn-HA, however, show promising data, as an increased adhesion and viability of osteoblasts was observed, compared to stoichiometric HA [54].

4.2. Pulsed Laser Deposition

Deposition of carbonated hydroxyapatite (CHA), magnesium-doped hydroxyapatite (Mg-HA) and octacalcium phosphate (Mg-OCP), strontium-doped apatite (Sr-HA) and carbonated hydroxyapatite (Sr-CHA), silicon doped-apatite (Si-HA), manganese-doped carbonated hydroxyapatite (Mn-CHA), and octacalcium phosphate (Mn-OCP) has been reported by PLD [39,46].

Deposition of carbonated hydroxyapatite allowed to obtain nearly stoichiometric films, Ca/P ratio increasing with deposition temperature (30 up to 700 °C) [43]. As for MS, also crystallinity degree and mechanical properties (Vickers microhardness) increase upon annealing [39]. Instead, no significant alterations in morphology are detected for annealing at temperatures below 500 °C, while they become pronounced at 700 °C and above.

Magnesium-doped hydroxyapatite (Mg-HA) at different magnesium content has also been deposited by PLD. Increasing Mg content was found to cause a parallel decrease in crystallinity and crystal size [40,55]. Effective incorporation of Mg in the lattice was verified. Significantly, Mg distribution is non-homogeneous and tends to form Mg-rich areas, where osteoblasts concentrate, attach and grow [40].

In vivo tests on rabbit models have been reported for both Mg-HA and magnesium doped octacalcium phosphate (Mg-OCP), having the same magnesium content (Mg: 0.6 wt.%) [56]. At sacrifice, 6 months after implantation, good bone growth and integration was found for both coated implants and for the uncoated titanium reference. No inflammatory reactions or failure were reported for any of the implants. Bone volume, bone-implant contact length and contact length ratio to implant surface has been found to be higher for Mg-HA and Mg-OCP implants compared to uncoated implants (μCT). Performances of Mg-HA were found to be higher than those of Mg-OCP, in our opinion also due to differences in solubility of the two compounds.

Strontium substituted hydroxyapatite (Sr-HA) [17] and strontium substituted carbonate apatite (Sr-CHA) [42] have been tested. Thin films of Sr-HA were obtained (200 nm up to 1 μm), that were annealed after deposition [17,42]. The presence of Sr in the coatings, which was verified by EDS, did not significantly impact on coatings morphology, as found elsewhere for plasma sprayed coatings [17].

In vitro, osteoblasts adhesion, proliferation, viability and differentiation were found to be boosted, the increase in adhesion (tensile adhesion by ASTM C633-01(2008) [57], ASTM F1147-05:2011 [58], ISO 20502:2005 [59]) since the early steps being proportional to Sr content [17]. Conversely, osteoclasts proliferation was found to be reduced, again depending on Sr content (3%–7%). Interestingly, spreading of osteoblasts is increased compared to stoichiometric HA coatings.

Deposition of Si substituted hydroxyapatite (Si-HA) has been reported, composition of the targets being successfully and finely transferred to the coatings [60]. In a study by Rau et al., it was found that the Si content must be kept below 2 wt.% to avoid a reduction in the decomposition temperature. This ultimately caused the formation of unwanted unstable phases, such as TCP and/or phosphate silicates [61]. Below 2 wt.%, instead, Si does not significantly alter HA lattice, as denoted by XRD spectra, almost identical to those of HA.

Addition of silica can cause an increase in the Ca/P ratio compared to the target, due to the formation of CaO [60,61]. This formation is relevant, as CaO is cytotoxic, hence compatibility of the coatings must be verified. Ca/P ratio is also dependent on the deposition temperature and/or on post-deposition annealing, as seen for MS [61]. Several temperatures have been tested in the literature for deposition of Si-HA (400, 500, 600, and 750 °C), all leading to deposition of dense and un-cracked films [61]. Films obtained at these temperatures are crystalline and nanostructured, crystallinity degree being obviously depending on annealing temperature. Interestingly, also thickness and Vickers microhardness are affected by deposition temperature [61]. However, despite mechanical properties generally increasing for increasing deposition temperature, when the temperature is brought above 750 °C, formation of byproducts (calcium-titanium mixed phases) hampers adhesion. Also, at these temperatures, Ca/P ratios completely different from those of the target (≈3.5) are obtained, indicating

HA decomposition alongside formation of byproducts. As a consequence, deposition temperatures around 500 °C are generally recommended.

In vitro mineralization is reported after 1 day of immersion in alfa-MEM, thickness of the HA-layer increasing for increasing soaking times (up to 28 days) [61].

Mn-substituted CaP coatings have been manufactured exclusively by PLD, to the authors' best knowledge. Deposition of both Mn-CHA and Mn-OCP coatings have been described in [62]. While Mn-CHA is more similar to real bone but has higher crystallinity and consequently slower dissolution rate, Mn-OCP is more soluble and is expected to experience faster dissolution—thus possibly being more effective in boosting bone growth at early stages. When tested in vitro, human osteoclasts and fibroblasts exhibited suitable adhesion, proliferation and viability onto both coatings, without any significant difference being assessed. This is ascribed by the authors to a compensation between the beneficial effects CO_3^{2-} of Mn-CHA, and the lower crystallinity of Mn-OCP.

These coatings were also validated in vivo in rabbit models, in comparison to HA coatings. Tensile strength by pullout test was tested at 8 weeks after implantation [63], indicating that Mn-OCP and, to an even higher extent, Mn-CHA, can better improve implant-bone integration with respect to HA, suggesting a higher osseointegration potential.

4.3. Deposition by Matrix Assisted Pulsed Laser Evaporation

Deposition of Sr-HA and Sr-OCP has been reported by Combinatorial Matrix-Assisted Pulsed Laser Evaporation (C-MAPLE).

The possibility to deposit metastable Sr-OCP and composites of hydroxyapatite and drugs (here alendronate and zoledronate), evidences the strength of this technique, that allows deposition of heat-sensitive material [27,64]. Sr-doping on OCP was found to improves cells proliferation, activity and differentiation and, hence, the overall behavior of the coating. Strontium zoledronate and HA composites, instead, were found to promote osteoblasts adhesion and boosts proliferation, compared to uncoated references.

4.4. Deposition by Pulsed Electron Deposition

Deposition of Sr-HA has been very recently reported by PED (in the novel Ionized Jet Deposition version) for variable Sr content (up to 9 wt.%) [36]. Deposition has been carried out at room temperature on a polymeric substrate (PEEK) leading to an increased in wettability and roughness compared to the bare substrate, and suitable mechanical properties. A good transfer of composition (including Sr content) has been assessed from the target to the deposited coatings [36].

5. Multi-Substituted Coatings and Deposition from Biogenic Sources

Multi substituted HAs have also been explored in the literature, to increase similarity to bone apatite and to exploit the capability of each ion to influence HA lattice, crystallinity and solubility, and the coatings mechanical properties and interactions with cells [24].

In this view, pulsed laser deposition of silicon and strontium co-substituted carbonated hydroxyapatite (SiSrCHA) has been reported [65]. SiSrCHA has been obtained by diatomaceous earth, naturally containing several further foreign ions (Fe, K, Mg, Na, and P), all possibly boosting biological performances [65]. In the study, silica has been kept constant (2.5 at.%), while Sr concentration has been varied in a wide range (0 up to 10 at.%). Deposition has been carried out at high temperature to tune crystallinity and adhesion. Mesenchymal cells adhesion, morphology and osteogenic differentiation and activity have been tested. Results showed that, for multi-substituted coatings, variations in the amount of one of the dopants might affect the content of the others. In fact, aside from an obvious decrease in Ca content, due to a progressive incorporation of Sr, increasing Sr also leads to alterations in carbonates content. This can be seen by a reduction in the intensity of carbonates bands in FT-IR and in the CO_3^{2-} to PO_4^{3-} ratio, compared to pure CHA. We ascribe that to the modifications caused by each substitution to the HA lattice, influencing its capability to host further foreign ions.

Instead, doping with Si and Sr does not influence cells morphology compared to pure CHA reference coatings, as good cells coverage is found at 28 days for all the examined group. Instead, increased cells proliferation and differentiation were found for the multi-doped coating, compared to references. We speculate that examining growth at lower times could be very useful to assess biological behavior.

A further, crucial step in the direction of obtaining coatings having increasing similarity to bone apatite is deposition directly from biogenic targets. Deposition of apatite coatings from targets from a biogenic source have been reported by PLD [66–69] and PED [33,34]. Deposition of doped bone apatite targets has also been reported [67,68].

In particular, by PLD apatite has been deposited starting from simple, LiO_2- and CIG-doped ovine and bovine bone, MgF_2, and Mg doped bovine bone, simple and titanium doped ovine dentine and human dentine. Further experiments about deposition of "natural apatite" by PLD have also been reported, tested in vitro and in vivo, but without suitable description of the apatite source and the deposition procedures [70,71].

Ovine and bovine bone-derived coatings were obtained by PLD, after calcination of the powders at 850°C for 4 h in air to remove organic components [66]. Characterizations performed on the targets, obtained by compression of the calcinated apatite powders, indicate the presence of brushite and phosphorous fluoride hydroxide traces, alongside HA. HA (calcium deficient) was also found to be the main phase in the coatings. Traces of Na, Mg, Cl, F and Si were detected in the coatings and the targets, suggesting that ion substitutions were preserved. No in vitro and/or in vivo biological tests are reported for these coatings.

HA powders from sheep dentine were also calcinated (6 h at 850° in air) to remove organic components and allow an easy separation between dentine and enamel [67]. After grinding and sieving to obtain particles of a suitable size, part of the powders was admixed with Ti powders. Both Ti-doped and un-doped powders were homogenized and pressed to obtain targets, later heated at 1000 °C for 4 h in air, and deposited by PLD.

Morphology and composition of the coatings were investigated, indicating that HA is the main phase in the coatings, alongside Ti minor phase and, possibly, $MgCO_3$ and $CaMg_2$, possibly resulting from calcination. Traces of Na, Mg, C and Ti (in the doped samples) were detected in the coatings by EDS [67].

Both coatings, respectively 1.8 microns (undoped) and 1.2 microns (doped) thick are composed of granular aggregates, providing a high surface roughness, suitable for cells adhesion. Addition of Ti also has an impact on the coatings morphology, as it causes a reduction in average grain size compared to undoped Ti and a denser particulate. Pull-out test (ASTM D4541-17 [72] and ISO 4624:2016 [73]) indicates a good bonding strength, increased in the case of Ti-doping (55.5 ± 5.5 MPa vs. 63.7 ± 5.2 MPa), due to Ti-Ca segregation at the interface between the coating and the substrate, creating a mingled interlayer. Biocompatibility was assessed by evaluating human mesenchymal stem cells (hMSC) morphology and proliferation, and toxicity assay, showing evidence of good biological behavior for both coatings, with and without titanium addition [67]. Bovine apatite from femoral bones was also used, alone and enriched with MgF_2 and MgO. As for ovine dentine, prior to deposition, bovine bone powders were calcinated (4 h at 850° in air), mixed, pressed in a mold and sintered (4 h at 1000 °C) to obtain the deposition targets. Post deposition annealing was performed to enhance crystallinity of the coatings [68].

Morphology, composition and adhesion (ASTM D4541-17 [72] and ISO 4624:2016 [73]) of the coatings were evaluated, then the influence of the targets and the coatings on HEp-2 cells viability was tested and gene expression and antimicrobial activity of the films were evaluated [68].

A close transfer of composition from the target to the coatings was discovered, with the absence of MgF_2 peaks and weak MgO peaks, that the authors attributed to Mg^{2+} and F^- substitution in the HA lattice. Peaks characteristic of CHA were shown by FT-IR. MgO and MgF_2 enhanced the

adhesion to the substrate and to provide anti-biofilm properties to the coatings. All films exhibited good biocompatibility, as none of them exhibited relevant cytotoxicity [68].

Human dentine, bovine-derived HA and ovine-derived HA were also deposited after calcination of the powders (4 h at 850°), pressing and sintering at 1100 °C for 4 h [69]. Deposition was performed at high temperature (500 °C) and post-treatment annealing at the same temperature (6 h, water vapor) was also carried out. Composition and crystallinity of the deposited coatings were investigated, showing a good transfer of substrate composition, including that of trace elements. Thickness of about 1.1–3.5 microns was obtained, depending on the precursor used. Pull-out tests (ASTM D4541-17 [72] and ISO 4624:2016 [73]) indicated a good adhesion to the substrate [68].

Bovine bone-deriving apatite has also been deposited by PED, including a more recent development of the technique called Ionized Jet Deposition (IJD) [33,34].

Bone apatite-like films were deposited directly from deproteinized bone targets, without any grinding or calcination. Deposition was performed at room temperature and half of the samples were heat treated after deposition, to improve crystallinity (1 h at 400 °C in air) [33,34].

Obtained films were compared to films obtained by sintered stoichiometric-hydroxyapatite targets with the same deposition procedure and parameters, both as deposited and annealed [33,34].

As-deposited and annealed coatings were characterized in terms of composition and crystallinity, microstructure and morphology, and mechanical properties (nanoindentation and micro-scratch, UNI EN ISO 20502:2005 [59]). Obtained coatings are nanostructured and exhibit sub-micrometric thickness. Results showed a close transfer of the targets' composition, including trace elements (Na and Mg and carbonate substitution). As-deposited coatings exhibited low crystallinity, that was significantly increased by post-deposition annealing, up to resembling that of biogenic apatite target. As a result of annealing, mechanical properties increased up to values comparable to those of commercial plasma-sprayed HA-coatings [34].

Biological tests are also reported, evaluating human dental pulp stem cells (hDPSCs) cells attachment, proliferation and the expression of typical osteogenic markers Runx-2, osteopontin, Osx, and Osteocalcin [33].

In vitro biological tests indicated that annealed bone apatite coatings promote hDPSCs proliferation to a higher extent compared to non-annealed bone coating and HA-references. Immunofluorescence and western blot analyses revealed that the typical osteogenic markers were expressed, suggesting that bone apatite-like coatings treated at 400 °C alone can efficiently promote the osteogenic commitment of the cells, even in absence of an osteogenic medium [33].

6. Conclusions and Future Perspectives

Plasma-sprayed (PS) coatings have extensively studied and used in clinical practice but, due some intrinsic limitations, their real effectiveness compared to uncoated implants is still controversial. Instead, good results have been so far obtained for novel plasma-assisted techniques, capable of depositing thin films. In fact, they were found to allow a more controlled transfer of stoichiometry of the deposited coatings and of ionic additions to plasma spraying. However, presence of secondary phases often reported for magnetron sputtering together with preferential sputtering of some ions (i.e., Ca instead of Sr), cause modifications in the Ca/P and substitution rate, and indicates an imperfect transfer of the target composition. Because relatively low changes in the composition of CaPs, especially when substituted can result in significantly different properties, we believe that it is a relevant limitation in the technique, which needs to be addressed. PLD, MAPLE and PED, instead, allow for a more precise transfer of the target composition to the coatings.

Crystallinity degree can also be finely tuned, by deposition at selected temperature or pre/post deposition annealing. Good adhesion and mechanical properties in general, were confirmed by all the examined studies, as well as good behavior in vitro in terms of adhesion, spreading, differentiation, morphology, and osteogenic activity of the cells, although not always higher than those of stoichiometric hydroxyapatite. Some promising results have also been described in vivo,

but extremely scarce literature on the topic and differences in trial conditions (animal model, trial time, characteristics of the bone, gap dimensions, etc.) make them scarcely conclusive.

Overall, thin films of ion-substituted apatites are promising materials, having good mechanical properties and biological behavior. Ion-substitution permits the availability of selected ions in selected concentrations in the peri-implant environment, with both a therapeutic effect and/or the capability to boost host cells response in view of the biomimetic principle. Deposition by plasma-assisted techniques allows to obtain thin films, having high adhesion and a fine control over crystallinity and composition, guaranteeing a tailored release.

Deposition from biogenic coatings has been emerging as a very promising alternative, as it allows to obtain multi-substituted coatings with ion-amounts and stoichiometry very close to those of real bone apatite, which is almost impossible to be achieved by artificial apatites. This new development has been made possible by PLD and PED, that allow a great control over the composition of the obtained targets. We believe that, once in vivo trials will be available for these coatings, this topic will obtain even wider interest and will expand its field of application.

Interestingly, however, despite ion-substituted coatings being generally proposed with the aim to better resemble real bone, mimicking of the real composition of bone is very limited, as generally only one or two ions are substituted simultaneously in doped HAs. In addition, there is a lack of correspondence between the concentrations of ions in biological HA and their biological relevance, and the number of studies devoted to their investigation. This can be partially ascribed to the wide variability in the concentration of trace ions in the bone, depending on the anatomical site, age, sex and to patient-specific characteristics such as, for example, diseases or metabolism. However, some ions, such as magnesium (highly present in bone and having significant biological relevance), is far less studied than Sr, whose concentration is significantly lower. Some other ions, such as Cl, Na and K are not even intentionally substituted. The number of studies, instead, seems more driven by the possible therapeutic role of the ions. This is clarified by intentional addition of specific therapeutic ions in biological apatite, which goes in the opposite direction of biomimetism. We believe that this, however, can be a promising route, further expanding the range of use of ion-substituted coatings.

Another important trend that is emerging regarding ion-substituted hydroxyapatite thin films, is that of antibacterial coatings. To this regard, a wide range, and increasing amount, of literature is available about silver-doped hydroxyapatite, for which deposition by magnetron sputtering [74–78], PLD [79–84] and MAPLE [85] has been reported. Being the study of plasma assisted deposition of Ag-doped hydroxyapatite of more recent introduction, the majority of the studies focus on the influence of deposition parameters on the films characteristics and on the evaluation of antibacterial efficacy, while extensive, comparative in vivo studies are yet to be performed. We believe that plasma-assisted deposition of apatites doped with a variety of ions having antibacterial (such as copper [86] and gallium), antitumor or other therapeutic actions (such as selenium [87] and cerium) will be increasingly pursued in the near future.

Overall, what we believe to be extremely significant is the possibility to pursue both aims, i.e., biomimetism and therapeutic functions, thanks to plasma assisted techniques.

We believe that extensive work still needs to be carried out to fully evaluate properties, potentialities and limitations of ion-doped apatite coatings. The main points that should be explored, in our opinion, are the following:

- A better understanding of the real benefits of ion-doped coatings, compared to un-doped HA. This is still controversial, mainly because effective incorporation of ions in the HA lattice is not always verified and, because direct comparison between doped and un-doped coatings with the same characteristics and deposited in the same conditions is generally non-available. This is important, as several parameters play a key role, together with the composition of the coatings.
- The effects of different ions should be compared, again considering the same deposition and post deposition conditions and/or the same coating properties.

- The number of experiments performed in vivo must significantly increase, as for some coatings, no in vivo literature is available at all.
- Different opinions are found about some parameters that complicate the evaluation of the coatings performance. Among the others, partial verses complete dissolution of the coatings should be addressed. The optimal properties to be preferred must be identified univocally.
- Based on the significant differences existing between different bone sources, a comparative evaluation between different precursors should be carried out.
- To the authors' best knowledge, no clinical trials are available on the topic [88]. Once research will be ready to move to the clinic, a clearer picture will be achieved on the effective potential of these coatings.

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