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Laponite nanoclays for the sustained delivery of therapeutic proteins

Ainhoa Gonzalez-Pujana [a,b,c], Manoli Igartua [a,b,c], Rosa Maria Hernandez [a,b,c,*], Edorta Santos-Vizcaino [a,b,c,*]

[a] NanoBioCel Research Group, Laboratory of Pharmaceutics, School of Pharmacy, University of the Basque Country (UPV/EHU), Paseo de la Universidad 7, Vitoria-Gasteiz 01006, Spain
[b] Biomedical Research Networking Centre in Bioengineering, Biomaterials and Nanomedicine (CIBER-BBN), Institute of Health Carlos III, Madrid, Spain
[c] Bioaraba, NanoBioCel Research Group, Vitoria-Gasteiz, Spain

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ABSTRACT

Protein therapeutics hold immense promise for treating a wide array of diseases. However, their efficacy is often compromised by rapid degradation and clearance. The synthetic smectite clay Laponite emerges as a promising candidate for their sustained delivery. Despite its unique properties allow to load and release proteins mitigating burst release and extending their effects, precise control over Laponite-protein interactions remains challenging since it depends on a complex interplay of factors whose implication is not fully understood yet. The aim of this review article is to shed light on this issue, providing a comprehensive discussion of the factors influencing protein loading and release, including the physicochemical properties of the nanoclay and proteins, pH, dispersion buffer, clay/protein concentration and Laponite degradation. Furthermore, we thoroughly revise the array of bioactive proteins that have been delivered from formulations containing the nanoclay, highlighting Laponite-polymer nanocomposite hydrogels, a promising avenue currently under extensive investigation.

1. Introduction

Protein and peptide therapeutics include growth factors, cytokines, enzymes, hormones or monoclonal antibodies. Such plethora of essential molecules are involved in the majority of biological processes, which makes them hold great promise in the treatment of a vast number of prevalent diseases, including endocrine disruptions, immune diseases or infections (Nie et al., 2021; Abune and Wang, 2021). Therapeutic protein products account with important advantages in comparison to their small molecule drug counterparts – which dominate in the pharmaceutical market – because of the possibility to produce them at large scale, their lower toxicity and their higher bioactivity and specificity (Abune and Wang, 2021; Zaman et al., 2019). Since the oral administration of protein / peptide products is hampered by the harsh conditions of the gastrointestinal tract, these therapeutics are predominately administered parenterally (Brown et al., 2020; Tong et al., 2020). Even via this route, the complex and delicate structure of protein products, their rapid degradation and renal clearance lead to a very limited half-life. This results in the need of multiple administrations, which hinders patient compliance and therefore, the efficacy of these therapies (Zaman et al., 2019). As a response to this limitation, numerous strategies have been explored to design formulations that obtain a controlled release of proteins (Nie et al., 2021; Abune and Wang, 2021; Sun et al., 2023; Zheng and Pokorski, 2021; Wu and Mu, 2020). However, important problems still arise, such as rough conditions within the fabrication methods that lead to the denaturation of protein products, significant initial burst release effects or fast and uncontrolled release kinetics (Abune and Wang, 2021; Bizeau and Mertz, 2021). Therefore, the design of advanced formulations that allow a controlled and sustained release of protein therapeutics still urges to date.

In such regard, nanoclays are nowadays being widely explored for sustained drug delivery (Gaharwar et al., 2019; Dong et al., 2021). Indeed, clay minerals emerge as robust candidates for the controlled release of proteins because of their biocompatibility, charged surfaces, swelling capacity and nanoscale characteristics (Katti et al., 2022; Tomas et al., 2018). They consist of alternating tetrahedral sheets, primarily composed of silicon and oxygen, and octahedral sheets, containing aluminum, magnesium, or other cations, along with hydroxyl groups. These layers can hold various cations or anions between them, contributing to the diverse properties and classifications of clay

* Corresponding authors at: Laboratory of Pharmaceutics, School of Pharmacy, University of the Basque Country (UPV/EHU); Paseo de la Universidad 7, 01006, Vitoria-Gasteiz, Spain.
E-mail addresses: rosa.hernandez@ehu.eus (R.M. Hernandez), edorta.santos@ehu.eus (E. Santos-Vizcaino).

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minerals. Attending to their specific structure, natural clay minerals are classified in different groups – e.g. smectite, kaolin, chlorite or illite – (Bergaya and Lagaly, 2013). However, their synthetic counterparts present important advantages such as control over the structure, composition and dimensions, superior material properties and enhanced purity (Bergaya and Lagaly, 2013).

Among the latter, the registered trademark product Laponite® describes a nanostructured synthetic smectite clay manufactured from hectorite-like natural minerals which was commercialized in the early 1960s by BYK Additives & Instruments (previously Laporte Industries) (Shafran et al., 2020). Clasically, Laponite has been used as a film forming agent and rheological modifier in myriad fields including ceramics, cosmetics, agriculture or household cleaning (BYK Additives, and Instruments, 2014). Because of its low cytotoxicity, its microorganism free nature and biodegradability, its use in the pharmaceutical industry has gained special attention, specially the Laponite XLG grade, a gel forming grade which presents low levels of heavy metals and minimum toxic effects (BYK Additives, and Instruments, 2014). Whereas the mechanical properties of Laponite have been widely explored for promoting angiogenesis and osteogenesis, its shear-thinning behavior has been employed to fabricate inks for printing and injectable materials (Chimene et al., 2020; Munoz-Perez et al., 2023; Rajput et al., 2022). Moreover, other characteristics of Laponite, including its adsorbability and swelling ability have been proven promising for drug delivery applications (Tomas et al., 2018; Kiaee et al., 2022; Stealey et al., 2023).

In this review article, we focus on the use of Laponite for sustained protein delivery. We first provide a comprehensive discussion of the main factors influencing protein loading and release from Laponite, including the physicochemical properties of the nanoclay and proteins, the surrounding pH, the composition of the dispersion buffer, the concentration of Laponite and proteins and the degradation of the nanoclay. Moreover, we revise in depth the bioactive proteins and peptides that have been delivered from Laponite to date. In particular, we discuss not only the results obtained with plain Laponite formulations, but also with Laponite-polymer nanocomposite hydrogels, which represent a promising alternative with multiple benefits that is nowadays being extensively explored.

2. Factors influencing protein loading and release from Laponite

2.1. Physicochemical properties of Laponite

As above mentioned, nanoclays offer the possibility to control the delivery of drugs, which is especially relevant in those that present a low stability such as proteins (Kiaee et al., 2022; Jaber et al., 2018). Laponite presents a 2:1 structure: it is composed of an octahedral sheet of magnesium oxide intercalated between two tetrahedral silica sheets (Brigatti et al., 2013). This unit cell is repeated many times in two directions, resulting in disc-shaped crystals of 0.92 nm in height and 25 nm in diameter that present a high aspect-ratio and surface area (Fig. 1A) (BYK Additives, and Instruments, 2014). In this structure, some Mg^{+2} ions are replaced by Li^{+}, which leads to the composition that typically presents the empirical formula $Na^{+}_{0.7}$ [$(Si_8\ Mg_{5.5}\ Li_{0.3})\ O_{20}\ (OH)_4]^{-0.7}$. As a result of this cation substitution, the faces of the Laponite discs present a net negative charge. On the other hand, the edges of the discs present small localised positive charges generated by adsorption of ions where the crystal structure terminates.

During clay manufacture, in the dry powder form of Laponite, the negative surface charges are balanced by Na^{+} ions that are adsorbed on the surfaces of the discs by electrostatic interactions (Becher et al., 2019). Adjacent crystals share Na^{+} ions, which results in a stacked arrangement of the discs (BYK Additives, and Instruments, 2014) (Fig. 1B). However, when the clay is dispersed in an aqueous solution, Na^{+} ions diffuse leading to the original net charges of the crystals. In this scenario, the weak positive charges on the edges of the discs interact with the negative charges of the faces, giving rise to a "house of cards" structure (BYK Additives, and Instruments, 2014). At low Laponite concentrations – typically under 1% according to the literature, but always dependent on multiple other factors such as ionic strength, pH, etc. – this leads to the formation of disperse nanoparticular Laponite aggregates that conform a sol network. At higher Laponite concentrations, "house-of-cards" interactions are significantly amplified, transitioning to a gel state (Kiaee et al., 2022; Jatav and Joshi, 2017; Ruzicka and Zaccarelli, 2011).

Laponite exfoliation and the subsequent "house of cards" structure formation gives rise to a wide range of potential interactions between the clay and therapeutic molecules. Therapeutics can be retained in the inter-layer sites, surface and edge sites, as well as in the inter-particle sites by means multiple mechanisms (Fig. 1C), all of which will depend on the surrounding pH and on the size and electrostatic properties of the therapeutic molecule (Kiaee et al., 2022; Aguzzi et al., 2007; Chiu et al., 2014; Das et al., 2016; Das et al., 2019; Aray et al., 2003). Interestingly, Laponite nanodisks can be chemically modified to broaden the range of molecules that can be attached to it. Covalent modification of the edges of the nanoclay has been proposed to introduce groups that enhance the loading and delivery of therapeutic agents (Tang et al., 2023; Mustafa et al., 2015).

Considering the above mentioned, Laponite can interact with multiple functional groups at different levels, showing a vast potential for the sustained and controlled delivery of therapeutic protein products. Indeed, studies showing the addition of serum supplemented cell culture media to Laponite significantly increased the hydrodynamic diameter and decreased the zeta potential, demonstrating that proteins are physically adsorbed onto the nanoclay (Carrow et al., 2018).

2.2. Physicochemical properties of proteins

Proteins are polymers formed by the alternation of a varying number of different amino acids joined by peptide bonds. Each protein presents a particular amino acid combination, and the specific nature of these building blocks will confer each protein unique physicochemical properties (Aftabuddin and Kundu, 2007). Native proteins are broadly categorized as hydrophilic or hydrophobic attending to their solubility, a crucial parameter in protein science (Navarro and Ventura, 2019; Qing et al., 2022). Whereas the former includes water-soluble proteins that reside mostly in cytoplasm, the latter comprises proteins embedded in membranes. Central to solubility are the polarity and charge of amino acids, which will dictate their ability to interact with water molecules. Examples of nonpolar amino acids are leucine (L), valine (V) or isoleucine (I), which cannot form any hydrogen bonds and therefore are hydrophobic. On the contrary, other amino acids, such as aspartic acid (D), asparagine (N) or glutamate (E), are hydrophilic because of their capacity to form hydrogen bonds with water molecules. Moreover, in the case of positively charged amino acids such as histidine (H), lysine (K) and arginine (R), not only can they form hydrogen bonds with water molecules, but they can be protonated at acidic or neutral pH values, which results in the formation of significant electrostatic interactions (Rossmann, 2009).

On the other hand, Laponite presents a high hydrophilicity, a good swelling ability and cation exchange capacity. Thus, protonated groups can strongly interact with the negative surface charges of Laponite and as a result, are more likely to be retained in the interdisc spaces. If as well as presenting a high positive charge, bioactive proteins show amino acids that form effective hydrogen bonds, a better retention capacity and therefore, a more sustained release will be obtained (Nie et al., 2021). Indeed, a recent article showed that protein charge was the main determinant for Laponite−protein interactions via electrostatic adsorption. Positively charged proteins interacted more strongly with the nanoclay, giving rise to larger Laponite−protein complexes than did negatively charged proteins. Thus, the protein charge controlled the release rate, observing a hindered diffusion and slower release in cationic proteins in comparison to those presenting a negative charge.

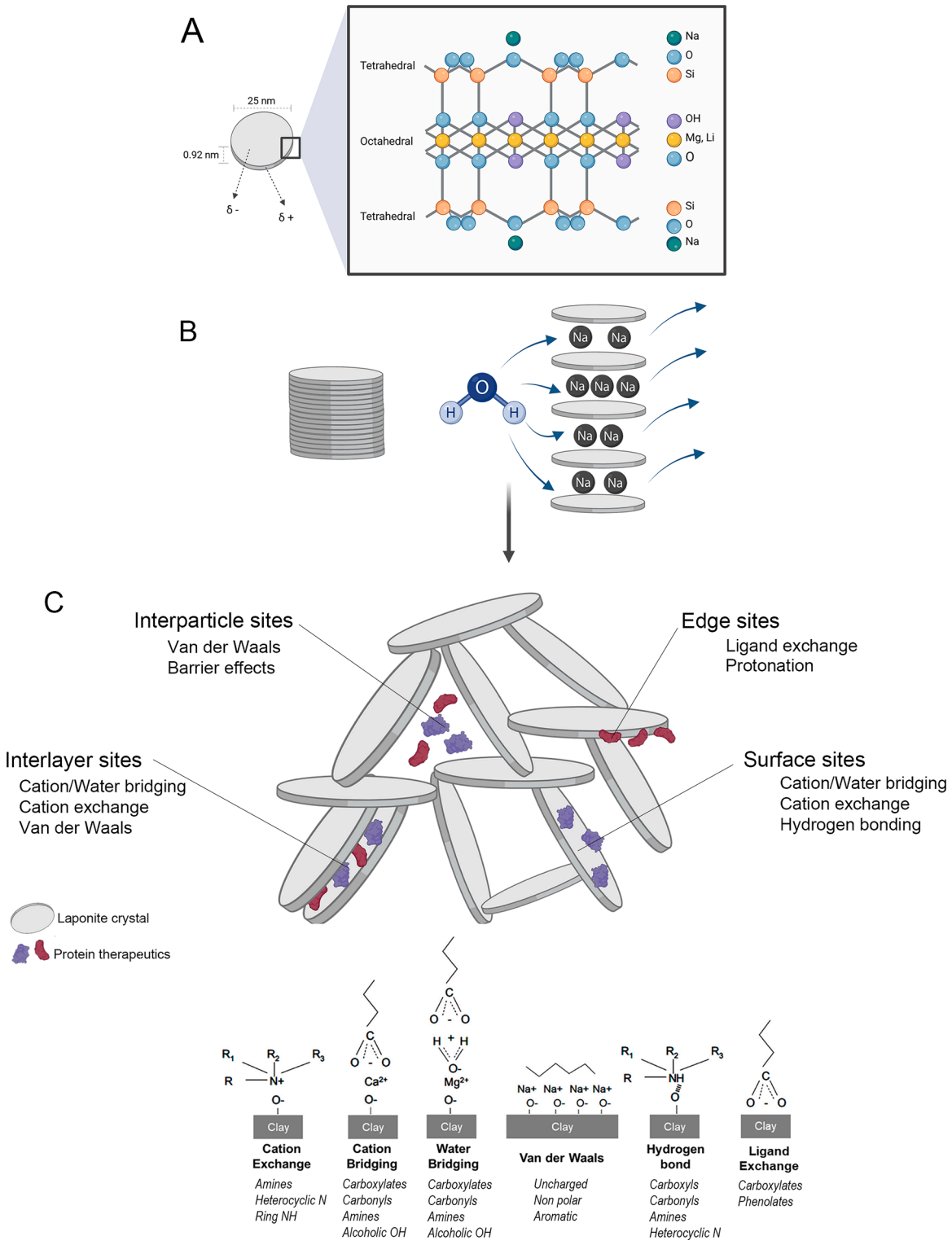

Fig. 1. Laponite structure and therapeutic protein loading. (A) Structure, shape, size and charge of Laponite crystals. (B) Stacked configuration of Laponite particles. (C) Laponite-therapeutic protein interactions in the "house of cards" structure.

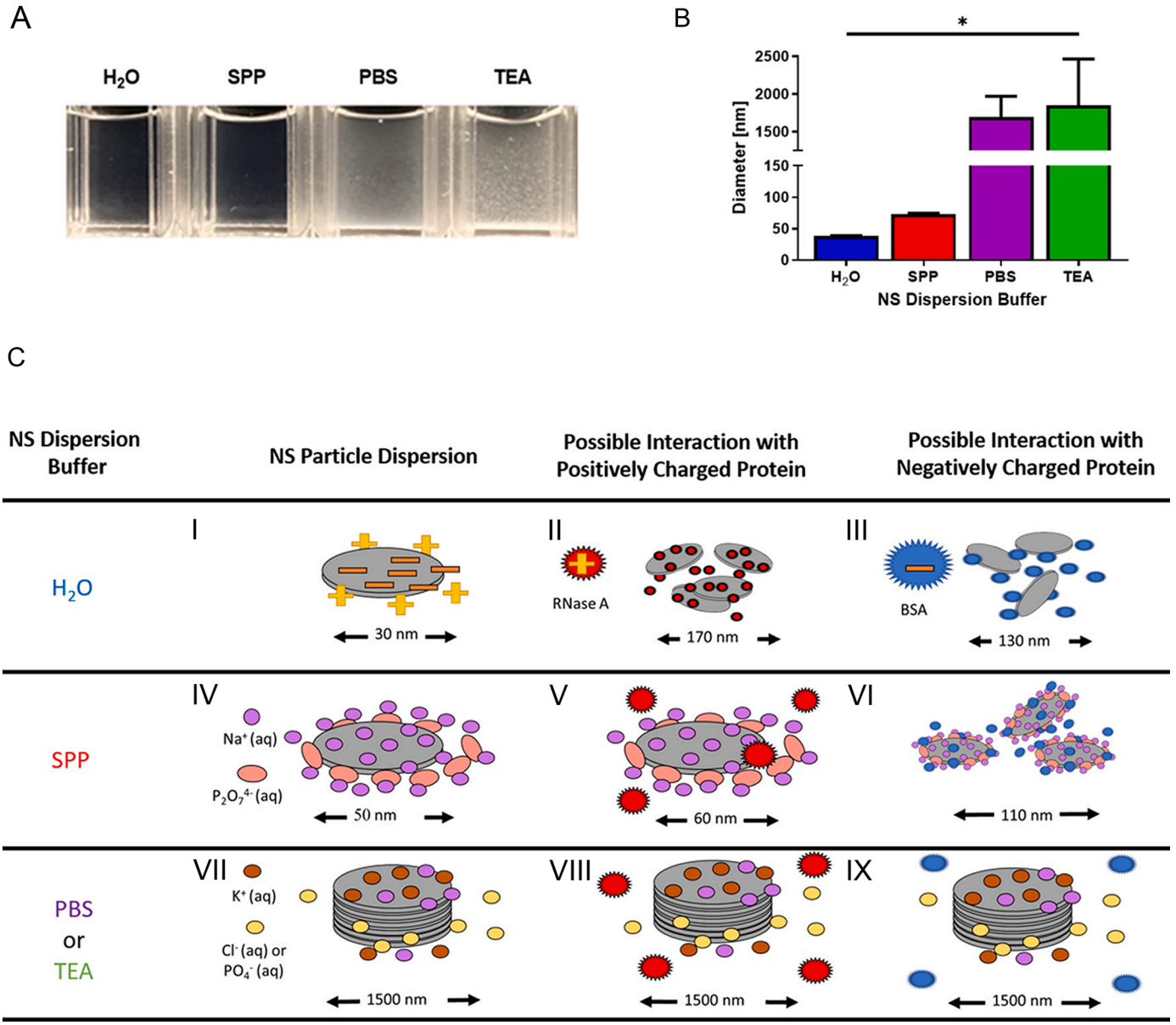

Fig. 2. Effect of the dispersion buffer composition. Effect of buffer solution on the dispersion of Laponite: (A) visual observation of dispersion of Laponite (10 mg/mL) in different buffer solutions, (B) Laponite (1 mg/mL) particle diameter in each buffer analyzed by dynamic light scattering. * indicates statistically significant difference between all samples ($n = 4$, $p < 0.05$). (C) Effect of buffer solution on Laponite – protein complexation. (I) In H_2O, Laponite was completely dispersed into individual particles with negatively charged faces and positively charged edges. (II) In the presence of positively charged proteins such as RNase, proteins electrostatically adsorb to the Laponite particle faces, forming Laponite – protein complexes, containing multiple Laponite particles. (III) Negatively charged proteins such as BSA will preferentially interact with Laponite particle edges, forming complexes that are typically smaller than those formed with positively charged proteins due to the difference in Laponite surface area available for binding. (IV) In SPP, Laponite was effectively dispersed due to a peptizing effect caused by the association of pyrophosphate ($P_2O_7^{4-}$) and sodium (Na^+) ions on Laponite particle edges and surface, respectively. (V) The SPP ion association forms an electrical double layer resulting in a charge shield that prevents adsorption of positively charged proteins. (VI) However, osmotic pressure in the form of unassociated ions can compress the thickness of this double layer, thereby allowing positively charged Laponite particle edges to interact with negatively charged molecules, including negatively charged proteins, forming a Laponite –protein complex. (VII) Neither PBS nor TEA was able to effectively disperse Laponite particles as tactoid structures persisted due to the relatively high osmotic pressures compared to H_2O and SPP. (VIII, IX) Because of this ineffective exfoliation, Laponite – protein complexes were unable to form with negatively or positively charged proteins. Adapted with permission from Stealey et al. (2021). Copyright 2021 American Chemical Society.

This fact has also been observed in non-proteic molecules, indeed, a recent article showed that negatively charged dextrans are released faster from Laponite than their positive counterparts, being both of the same size (Munoz-Perez et al., 2024). Despite these differences in the release rate, it is important to mention that overall, independent to their charge, Laponite is able to decrease their burst release and slow their delivery profiles, in comparison to formulations without the clay (Stealey et al., 2021). This is explained because anionic proteins can bind the edges of the discs and also present surface patch binding, in which their anisotropic charged surface contains regions where the surface charge is more positive, which allows them to interact with the negatively charged surface of Laponite discs.

Since the adsorption of proteins to Laponite is importantly governed by electrostatic interactions, the competition between the protein product and the surrounding molecules will dictate not only the loading efficiency but also the drug delivery. A recent study introduced the use of BSA as a displacement strategy to enhance the release of adsorbed molecules from Laponite, overcoming the important issues of low drug release from the nanoclay (Munoz-Perez et al., 2024). This same study demonstrates the possibility to load and release 150 kDa dextrans from Laponite, emphasizing the potential to deliver large protein molecules such as antibodies.

2.3. pH

As already stated, ionic forces are key for drug-Laponite interactions. Therefore, factors influencing the net charge of both, the nanoclay and the target protein will have a significant effect in the loading and release, being critical factors to bear in mind when designing the carrier systems (Kiaee et al., 2018). The main factor influencing these charges is the pH. In the case of Laponite, while the surface of the discs is permanently negative, the edges are pH sensitive, since according to the surrounding acidity or alkalinity the hydroxyl groups can be protonated or deprotonated, altering the net charge (Kiaee et al., 2018; Choi et al., 2021; Jansson et al., 2019; Wang et al., 2016). This confers the nanoclay a significant pH-buffering ability (Thompson and Butterworth, 1992; Jatav and Joshi, 2014). The isoelectric point of Laponite is pH ~ 10 (Tawari et al., 2001), thus, at lower pH values the edges are positively charged, whereas higher pH numbers favor deprotonation, giving rise to a negative charge (Jansson et al., 2019).

Considering this, in terms of drug loading, peptides/proteins presenting a negative charge could present a favored intercalation at lower pH values, since, at this condition, the higher positive charge of the edges of Laponite favors the electrostatic interactions. However, this assumption will not always take place, since pH will at the same time affect the target protein. Thus, acidic pH values below the isoelectric point of the protein will make the charge positive and it will therefore present more affinity for the surface of the discs. Something similar occurs regarding the release. Proteins presenting a positive charge may be released faster in acidic conditions since, in this scenario, H^+ will substitute the electrostatically loaded cationic drug. However, it has to be considered that again, that for the protein to present such charge, the pH values must be below its isoelectric point (Xiao et al., 2016).

The pH-responsiveness of Laponite can result specially relevant considering that the physiological pH can vary in particular pathological conditions. For instance, it is possible to take advantage of the acidic pH environments typically observed in inflamed, neoplastic or ischemic tissues to obtain and active release of the drug at the target site (Chen et al., 2022; Gan et al., 2020; Toft et al., 2021; Tóth et al., 2020).

2.4. Dispersion buffer composition

The ionic strength of the dispersion buffer is another important factor to consider. Effectively dispersing Laponite particles is key to maximize the surface area available for proteins to electrostatically interact with the clay. The stability of Laponite dispersions depends on the critical coagulation concentration (CCC) or critical coagulation ionic strength (CCIS), the threshold limit of electrolyte concentration above which particle aggregation occurs and the dispersion is destabilized (Elimelech et al., 1995; Galli et al., 2020). Ionic strengths above the CCC impede the formation of the "house of cards" structure and lead to particle aggregation (Kiaee et al., 2022; Liu et al., 2022). Such agglomeration minimizes the surface area of Laponite and therefore, hinders protein adsorption, and at the same time obstructs the release of molecules already attached to the clay.

A recent publication depicts this by studying the effect that different buffer compositions have on the dispersion of Laponite (Stealey et al., 2021) (Fig. 2). In particular, they selected a physiological buffer: phosphate- buffered saline (PBS); a buffer expected to exfoliate Laponite particles: sodium pyrophosphate (SPP); a buffer promoting Laponite-polymer hydrogel formation: triethanolamine (TEA) and deionized water (H_2O). As expected, PBS and TEA failed to disperse Laponite particles because of their high ionic strength (Fig. 2 A, B) (Jatav and Joshi, 2014; Sheikhi et al., 2018). Importantly, this study also showed the effect that the dispersion buffer had over protein loading and release (Fig. 2C). In PBS and TEA dispersions, Laponite failed to load proteins since the clay was not dispersed but formed a stacked structure that presented a minimal surface area for the proteins to electrostatically interact. In the case of SPP, it has to be considered that pyrophosphate anions shield Laponite edges, leaving the whole clay particle with a negative charge that attracts Na^+ cations around the particles (BYK Additives, and Instruments, 2014). These positive charges lead to a minimal interaction of the clay with cationic proteins, effect that was not observed in negatively charged proteins. On the contrary, using H_2O to disperse the clay led to strong interactions with cationic and anionic proteins, since Laponite was well dispersed and charge shielding did not occur. Additionally, H_2O was the one sustaining to a major extent the protein release and thus, considered the most efficient vehicle tested (Stealey et al., 2021).

2.5. Protein and Laponite concentration

The concentration of the protein therapeutic and the nanoclay will directly influence the loading. The personal care grade Laponite XLG presents a cation exchange capacity around 60 meq per 100g according to the manufacturer (BYK Additives, and Instruments, 2014). Considering that the milliequivalent weight of Na^+ is 23 mg/meq, 100g of the nanoclay can intercalate up to 1.38 g of Na^+ (Ghadiri et al., 2013). Assuming that a complete Na^+/drug exchange can take place – which could be compromised in the case of large proteins due to steric hindrance –, it is possible to calculate the maximum amount of the therapeutic protein that can be intercalated considering its molecular weight. If its concentration is above the maximum loading capacity of Laponite, the encapsulation efficiency will be reduced. Ghadiri *et al.* proved this using tetracycline, a drug that has been extensively studied for Laponite loading. According to its molecular weight, 444.4 g/mol, 26.7 g of the drug can be intercalated in 100 g of the nanoclay. In their studies, increasing the drug concentration from 0.1 wt % to 0.3 wt % reduced the encapsulation efficiency from 99.2% to 95.5% (Ghadiri et al., 2013).

Regarding the concentration of Laponite, there has been intense debate around its effect over drug loading. Some authors defend that increasing the quantity of the nanoclay leads to a better drug dispersion within its layers and therefore, increases the encapsulation efficiency (Xiao et al., 2016). However, other publications state that increasing Laponite levels could cause the aggregation of the nanoclay discs. In this scenario, the drug intercalation would be compromised, having a detrimental effect over drug loading (Kiaee et al., 2022; Wang et al., 2012). The contradictory results obtained may be due to the intrinsic nature of the drug employed and therefore, preliminar studies with specific therapeutics could shed light on the adequate clay concentration to use in each case. When considering this factor, it is also important to bear in mind that Laponite concentration will also have an important

effect over drug release. Higher clay concentrations lead to a lower drug diffusion as well as to a slower clay degradation rate (Waters et al., 2016; Pacelli et al., 2016).

2.6. Swellability and degradability of Laponite

The high swelling behavior of Laponite makes it attractive for drug loading, since it directs the exfoliation process that enables to load therapeutics by the mechanisms above cited (BYK Additives, and Instruments, 2014; Jiang et al., 2022). Moreover, in the case of Laponite hydrogels, drug release kinetics are directly influenced by the swelling, since when increasing, it leads to a greater hydrogel mesh size that results in a faster release. Therefore, factors influencing the swelling would also have an impact in protein loading and release (Valencia et al., 2018). Among them, the ionic strength of the surrounding media can considerably weaken the swelling abilities (Li et al., 2009). It should present values below the already described CCIS in order to avoid particle aggregation, which hinders exfoliation and therefore, drug loading (Elimelech et al., 1995; Galli et al., 2020). Another factor is the pH. As the pH increases and more hydroxyl groups deprotonate, the negative charge density on the Laponite layers increases. This leads to greater electrostatic repulsion between adjacent Laponite layers. The increased negative charge density enhances the attraction of water molecules, causing the layers to swell apart (Li et al., 2009). Laponite concentration has also been reported to impact swelling, however, mixed results have been published. In particular, with regard to Laponite hydrogels, some studies report that high concentrations of the nanoclay result in a more hydrophilic system and infiltration of water molecules. Other studies contradict these results claiming that high concentrations of Laponite lead to a higher crosslinking degree, which hinders the penetration of water and therefore, the swellability (Li et al., 2009; Waters et al., 2018). In aqueous Laponite dispersions, the aging is another factor to consider since aged solutions present an increased viscosity that limits water diffusion. The contrary occurs with temperature, with a decrease of viscosity at high temperatures, which increases the swelling. Heat treatment of Laponite hydrogels has also shown a significant improvement in the values of water absorption capacity (Li et al., 2009).

Laponite degradation has been demonstrated a key step for protein release (DuBose et al., 2005). As a biodegradable nanoclay, Laponite naturally dissociates into aqueous silica ($Si(OH)_4$), sodium, magnesium and lithium ions when the environmental pH values are below the isoelectric point of the clay (pH ~10) (Thompson and Butterworth, 1992; Brokesh et al., 2024). Under these conditions, H^+ ions interact with Laponite, which leads to the leaching of Mg^+ and Li^+ ions and thus, to the biodegradation of the nanoclay in around 20–50 days (Jatav and Joshi, 2014; Mohanty and Joshi, 2016). To estimate the degradation of the clay it is possible to correlate the amount of released Mg^{2+} to the dissolution rate of Laponite considering the following formula (Thompson and Butterworth, 1992):

$$Si_8Mg_{5.45}Li_{0.4}H_4O_{24}Na_{0.7} + 12H^+ + 8H_2O \rightarrow 8Si(OH)_4 + 0.7Na^+ + 5.45Mg^{2+} + 0.4Li^+$$

It has been reported that *in vivo*, cells internalize nanoclay particles by clathrin-mediated endocytosis, which is engulfed in endosomes and degraded within their low pH values (Carrow et al., 2018; Brokesh et al., 2024; Iturrioz-Rodríguez et al., 2021). It is important to highlight that Laponite dissociation products have been demonstrated cytocompatible, enabling normal cellular metabolism and proliferation over time (Brokesh et al., 2024). Different factors influence the degradation of Laponite and can be modified to control the drug release. Among them, it has been reported that an increase in the concentration of Laponite in the formulation presents a stabilizing effect against degradation (Table 1) (Mohanty and Joshi, 2016). An interesting study investigated the degradation rates of Laponite/alginate composite hydrogels with varying composition ratios. Results indicated that after 48h, 20:80 and 50:50 (Laponite/alginate) hydrogels exhibited mass losses of 48% and 28%, respectively. Outstandingly, high clay mineral concentrations (80:20) demonstrated a significantly lower degradation rate (5.5% in 48 h) (Ghadiri et al., 2013). In another example, Laponite/poly(ethylene oxide) nanocomposites containing 40% to 70% of Laponite were incubated in PBS for 21 days. Again, the same tendency was observed: the mass loss decreased from 47% to 23% by increasing the Laponite content (Gaharwar et al., 2011).

Table 1
Examples exhibiting the influence of Laponite content in formulations over degradation (Ghadiri et al., 2013; Gaharwar et al., 2011).

Laponite/polymer ratio	Time (days)	Mass loss (%)
20:80	2	48
40:60	21	47
50:50	2	28
70:30	21	23
80:20	2	5.5

3. Sustained release of proteins from Laponite

The charged nature of Laponite, together with its high surface area renders the nanoclay a promising vehicle to adsorb large proteins and small peptides. As already outlined, the strong electrostatic interactions with the nanoclay can lead to a sustained delivery with minimal burst release for both, cationic and anionic protein therapeutics (Stealey et al., 2021; Gaharwar et al., 2019; Kim et al., 2020). The delivery of different molecules of proteic nature has been studied in recent years, especially of growth factors and cytokines (Nexprot, 2023). Despite it is possible to load protein therapeutic products into plain Laponite formulations (Black et al., 2022; Cross et al., 2019; Gibbs et al., 2016; Chen et al., 2018; Chen et al., 2017; Chen et al., 2016; Page et al., 2019), the majority of studies evaluate the incorporation of the nanoclay to polymeric hydrogels to form nanocomposite materials with prolonged release properties (Waters et al., 2016; Waters et al., 2018; Kim et al., 2020; Wu et al., 2022; Kilian et al., 2022; Zhang et al., 2020; Chang et al., 2023; Koshy et al., 2018; Zandi et al., 2021; Howell et al., 2018; Ding et al., 2016; Wang et al., 2019; Li et al., 2018; Quint et al., 2022; Mohammadi et al., 2022; Li et al., 2023; Cao et al., 2023; Erezuma et al., 2022; Saygili et al., 2022; Zhang et al., 2020; Ahlfeld et al., 2017; Dawson et al., 2011; Quint et al., 2021) (Table 2).

3.1. Laponite formulations

As previously discussed, Laponite can load and release a plethora of bioactive proteins for therapeutic purposes. Among these proteins, the study of Laponite gels to control the release of osteogenic factors stands out, specially the bone morphogenetic protein 2 (BMP-2). This growth factor has been extensively employed for bone induction. However, the majority of approaches tested to administer BMP-2 in a controlled fashion reach supraphysiological doses that have been described to cause important adverse effects such as heterotipic tissue formation, osteolysis and inflammation (Cheng et al., 2018). These high doses are usually the result of an initial burst release. In this sense, the delivery of BMP-2 by means of Laponite gels has demonstrated to substantially reduce this effect and therefore, the effective dose to use. Indeed, osteogenic differentiation *in vitro* has been achieved at around 30000-fold lower doses than those used in the clinical practice; whereas ectopic bone formation *in vivo* has been promoted at one order of magnitude below the minimum effective doses recorded in the literature (Gibbs et al., 2016) (Fig. 3).

Other studies corroborate these results, showing that electrostatically binding protein therapeutics such as BMP2- or transforming growth factor β 3 (TGF-β3) to nanosilicates prolongs their release, promoting their inductive ability and allowing to reduce the dosing (Cross et al., 2019). These studies showed that the sustained delivery of BMP-2

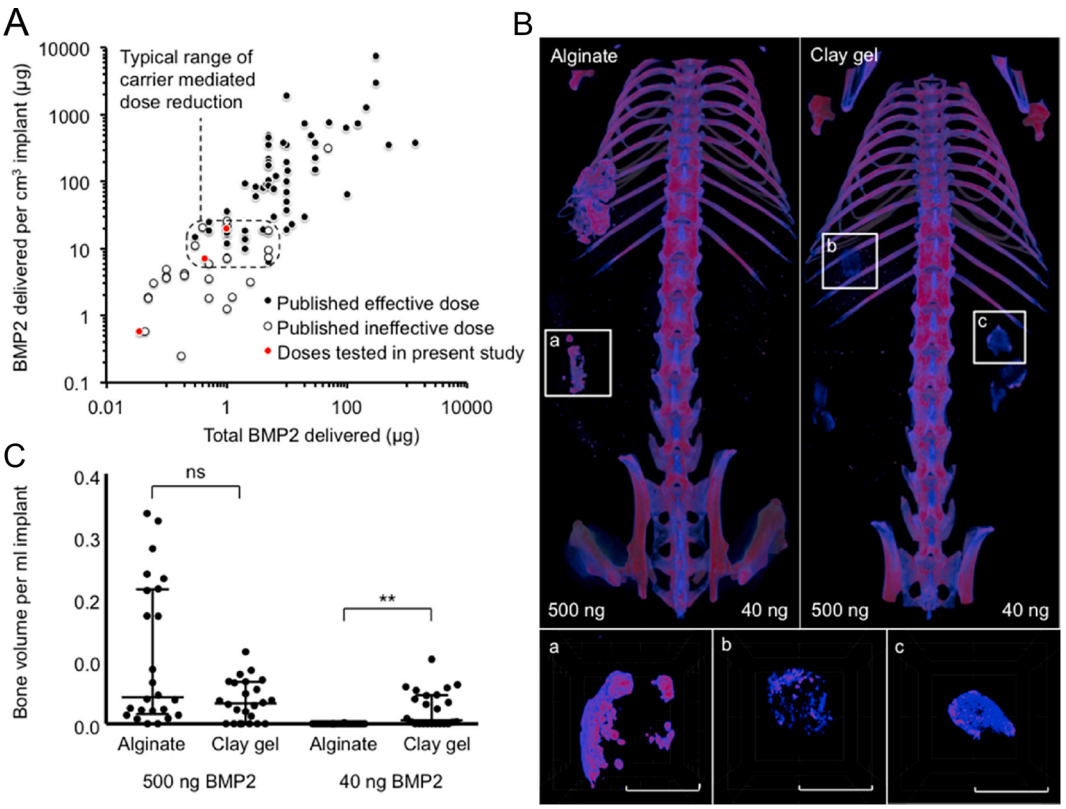

Fig. 3. BMP-2 delivery from Laponite gels. Clay gels uniquely sustain ectopic bone formation at low doses of BMP2. (A) Effective and ineffective BMP-2 doses tested for ectopic bone induction reported in literature. Total dose against dose per cm3 implant derived from 67 identified studies testing 72 carrier materials for BMP-2 induction of ectopic bone (subcutaneous or muscle) across a range of animal models. (B) False color mCT reconstructions of ectopic bone through Laponite or alginate delivery of encapsulated 500 ng and 40 ng doses of BMP-2 (n = 24) perfused through a collagen sponge. Scale 2.5 mm. (C) Laponite delivery sustained significantly higher ectopic bone formation at 40 ng doses of BMP2 compared with alginate control. N = 24, ** indicates $p < 0.01$, Kruskal-Wallis with Dunn's multiple comparisons test. Graph plots median with interquartile range. Reprinted from Gibbs et al. (2016), with permission from Elsevier.

is able to enhance osteogenic differentiation, whereas binding TGF-b3 to Laponite results in the chondrogenic differentiation of human mesenchymal stromal cells (MSCs) at lower concentrations than the exogenously administered factors. These results show that the proteins are bound to the nanoclay without altering their conformation and maintaining their efficacy (Cross et al., 2019).

Interestingly, Laponite formulations have also been employed to deliver in a controlled fashion vaccine adjuvants of proteic nature. Laponite has been proven to efficiently load antigens and promote strong cellular and humoral immune responses (Chen et al., 2018; Chen et al., 2017; Chen et al., 2016). Such is the case of Intimin b (IB) an outer-surface membrane protein that plays a key role in the infection caused by *Escherichia Coli*. Studies in mouse models showed that IB-loaded Laponite nanoparticular sol formulations were able to facilitate the uptake by immune cells and induce higher IgG levels than the commercially available potent adjuvant QuilA, after two subcutaneous injections of vaccine formulation (primary injection and boost injection 3 weeks later) into the nuchal region (Chen et al., 2016). Moreover, high IgG levels were maintained up to 4 months because of the long-term depot effect (Chen et al., 2016). Additional studies report the capability of Laponite nanoparticles to load multiple antigens – IB, proprietary antigen 1 and proprietary antigen 2 – which are also able to elicit significant mucosal, humoral and cellular immune responses, when subcutaneously implanted in mice, that prevent bacteria from adhering to mammalian cells (Chen et al., 2018) .

3.2. Laponite-polymer nanocomposite hydrogels

Polymeric hydrogels have been thoroughly studied for the sustained release of therapeutics, including bioactive peptides and proteins (Gonzalez-Pujana et al., 2022; Gonzalez-Pujana et al., 2020). However, one of the drawbacks of using hydrogels as delivery systems is their susceptibility to suffer an initial burst release because of their highly porous structure (Vigata et al., 2020). Among the different strategies to overcome this issue, the inclusion of nanoclays into the polymeric network represents a promising approach. Being highly hydrophilic, Laponite presents the ability to interact with a wide variety of polymeric matrices, which offers multiple benefits. First, the adsorption of therapeutics to the clay importantly limits the burst release, allowing a controlled and sustained delivery. Moreover, Laponite is able to improve the shear-thinning properties of hydrogels, which is paticularly relevant to perform a localized and minimally invasive administration (Samimi Gharaie et al., 2018). Indeed, Laponite has demonstrated to confer good printability and stability to hydrogels which do not meet the necessary flow characteristics for their semi-solid extrusion-based 3D printing (Munoz-Perez et al., 2023). Further, as well as enhancing the mechanical properties and physical stability of hydrogels, Laponite is also able to crosslink polymers such as hyaluronic acid (HA) (Kim et al., 2020). While the addition of Laponite to hydrogels presents important advantages, it is also important to underline that polymeric hydrogels also complement the effect of Laponite, since the nanoclay can offer a prolonged release by itself but may be rapidly cleared from the body. Importantly, the US Food and Drug Administration (FDA) has recently given the 510k approval to Laponite-loaded polymeric hydrogels, which demonstrates biocompatibility and highlights the clinical potential of this strategy (U.S. Food, and Drug Administration, 2022).

When fabricating Laponite-polymer nanocomposite hydrogels, a key factor is the protocol followed for drug loading. Different publications in

Table 2
Protein therapeutics delivered by means of Laponite formulations or Laponite-polymer nanocomposites.

Protein	Protein pI	Protein MW (kDA)	Carrier	References
Laponite formulations				
BMP-2	9.15	44.70	Laponite	(Black et al., 2022; Cross et al., 2019; Gibbs et al., 2016)
Intimin-b	8.7	94.00	Laponite	(Chen et al., 2018; Chen et al., 2017; Chen et al., 2016)
TGFb3	8.31	47.33	Laponite	(Cross et al., 2019)
VEGF	9.21	27.04	Laponite	(Page et al., 2019)
Laponite – polymer nanocomposites				
Anti-PD-1	-	32.00	Gelatin – Laponite	(Wu et al., 2022)
Angiogenin	9.73	16.55	Gelatin methacrylate – Laponite	(Waters et al., 2016)
			Gelatin – Laponite	(Waters et al., 2018)
BMP-2	9.15	44.70	Hyaluronic acid – Laponite	(Kim et al., 2020)
			Alginate-methylcellulose – Laponite	(Kilian et al., 2022)
			Hyaluronic acid-dextran – Laponite	(Zhang et al., 2020)
			Gelatin methacrylate – Laponite	(Waters et al., 2016)
BMP-4	8.97	46.55	Hyaluronic acid methacrylate – Laponite	(Chang et al., 2023)
CCL20	9.21	10.76	Alginate – Laponite	(Koshy et al., 2018)
EGF	5.53	133.99	Gelatin metacryloyl – Laponite	(Zandi et al., 2021)
Endothelin-1	9.52	24.42	Gelatin – Laponite	(Waters et al., 2018)
Endostatin	5.45	15.40	Gelatin – Laponite	(Waters et al., 2018)
FGF2	10.54	22.62	Collagen – Laponite	(Howell et al., 2018)
			Heparin – Laponite	(Ding et al., 2016)
			Gelatin methacrylate – Laponite	(Waters et al., 2016)
FGF4	9.73	22.05	Heparin – Laponite	(Wang et al., 2019)
Flt3L	7.6	26.42	Alginate – Laponite	(Koshy et al., 2018)
GM-CSF	5.21	16.30	Alginate – Laponite	(Koshy et al., 2018)
HGF	8.22	83.13	Gelatin – Laponite	(Waters et al., 2018)
IGF-1	9.78	21.84	Alginate – Laponite	(Li et al., 2018)
			Gelatin methacrylate – Laponite	(Quint et al., 2022)
IL-2	7.67	17.63	Alginate – Laponite	(Koshy et al., 2018)
IL-10	8.19	20.50	Gelatin methacrylamide – Hyaluronic acid methacrylamide – Laponite	(Mohammadi et al., 2022)
IL-15	5.13	18.09	Alginate – Laponite	(Koshy et al., 2018)
PDGF	9.39	27.28	Collagen – Laponite	(Howell et al., 2018)
			Gelatin methacrylyol – Laponite	(Li et al., 2023)
			Methacrylated gelatin – Methacrylated alginate – Laponite	(Cao et al., 2023)
PTX3	4.94	41.98	Gelatin – Laponite	(Waters et al., 2018)
SDF-1	9.92	10.67	Hyaluronic acid – Alginate – Laponite	(Erezuma et al., 2022)
TIMP-1	8.46	23.17	Gelatin – Laponite	(Waters et al., 2018)
TGF-β	8.31	47.33	Alginate – Polyacrylamide – Laponite	(Saygili et al., 2022)
			Methacrylated gelatin – Methacrylated alginate – Laponite	(Cao et al., 2023)
VEGF	9.21	27.04	Alginate – Laponite	(Zhang et al., 2020)
			Alginate methylcellulose – Laponite	(Ahlfeld et al., 2017)
			Collagen – Laponite	(Dawson et al., 2011)
			Collagen – Laponite	(Howell et al., 2018)
			Gelatin methacrylate – Laponite	(Quint et al., 2021)
			Methacrylated gelatin – Methacrylated alginate – Laponite	(Cao et al., 2023)
			Gelatin methacrylate – Laponite	(Waters et al., 2016)
			Gelatin – Laponite	(Waters et al., 2018)

Protein isoelectric point (pI) and molecular weight (MW) were obtained from Nexprot (2023). Anti-PD-1: anti-programmed cell death protein 1. BMP-2: bone morphogenetic protein 2. BMP-4: bone morphogenetic protein 2. CCL20: C-C motif chemokine ligand 20. EGF: epidermal growth factor. FGF-2: fibroblast growth factor 2. FGF-4: fibroblast growth factor 4. Flt3L: FMS-like tyrosine kinase 3 ligand. GM-CSF: granulocyte-macrophage colony-stimulating factor (GM-CSF). HGF: hepatocyte growth factor. IGF-1: insulin-like growth factor 1. IL-2: interleukin 2. IL-10: interleukin 10. IL-15: interleukin 15. PDGF: platelet-derived growth factor. PTX-3: pentraxin-related protein. SDF-1: stromal cell-derived factor 1. TIMP-1: tissue Inhibitor of Metalloproteinase 1. TGF-β: transforming growth factor β. VEGF: vascular endothelial growth factor.

the literature claim significant differences in drug release when incubating the therapeutic protein with the clay prior to adding the hydrogel forming polymer. This pre-incubation allows clay-protein complexes to form and constitutes another barrier to sustain the drug release from the nanocomposite hydrogels (Stealey et al., 2023; Stealey et al., 2021; Koshy et al., 2018). This is related to the fact that when fabricating the nanocomposites clay-polymer and clay-protein interactions occur simultaneously. Traditionally, in studies using Laponite to optimize the mechanical properties of a hydrogel, polymer-clay interactions are favored and the capacity of the clay to sustain the release of therapeutics is left behind. On the contrary, in applications where prolonged release of bioactive molecules is seeked, the contribution of the nanoclay to enhance the physical properties of the hydrogel is minimal (Li et al., 2018; Jafarbeglou et al., 2016). This can be explained because both strategies predominantly employ the same interaction site, which are the negatively charged surfaces of the Laponite discs. However, recently, interesting approaches are being considered to seize all the benefits that Laponite can offer. Kim et al. took advantage of the specific bisphosphonate interactions that occur with the positively charged disc edges of Laponite to fabricate self-assembling hydrogels that preserve the maximum surface exchange capacity of the nanoclay. In particular, they tethered bisphosphonate groups – analogs of pyrophosphates – to hyaluronic acid (HA) polymers. When HA was mixed with Laponite, the clay edges were bound to the bisphosphonate groups, preserving the whole negatively charged surfaces for additional functionality, in this case, protein loading (Kim et al., 2020).

Among the therapeutic proteins loaded into Laponite-polymer nanocomposite hydrogels, vascular endothelial growth factor (VEGF) stands out for inducing angiogenesis. Among different approaches, alginate-Laponite microspheres have been shown to sustain the release of VEGF for 28 days, maintaining the bioactivity of the growth factor. *In vivo*, cell laden alginate-Laponite microspheres loaded with VEGF significantly promoted tissue regeneration and angiogenesis, with the formation of new micro-vessels. Encapsulated cells maintained their viability above the 85%, proving the biocompatibility of the nanoclay (Zhang et al., 2020). VEGF loading in Laponite hydrogels demonstrated that the release of the growth factor dramatically changed to a more prolonged delivery rate in comparison to the same hydrogels without the nanoclay (Ahlfeld et al., 2017). In another strategy using 3D-printing, VEGF-loaded gelatin-methacrylate-Laponite hydrogels were directly printed *in vivo* in the skeletal muscle of a murine model of volumetric muscle loss injury. The slow release of VEGF increased $CD31^{+}$ capillaries, reduced fibrosis and improved muscle functional performance, demonstrating the potential of the strategy for the treatment of soft tissue traumas (Quint et al., 2021). Taking advantage of the high surface area of the nanoclay, the delivery of VEGF from Laponite-polymer hydrogels has been also combined with other factors. Such is the case of BMP-2 for bone-defect models. Collagen-Laponite hydrogels loaded with both growth factors were implanted in mouse femur segmental defects and results showed a significantly higher vessel volume after 4 weeks in comparison to the control group without Laponite (Dawson et al., 2011). In this line, collagen-Laponite gels loaded with VEGF, fibroblast growth factor (FGF) and platelet-derived growth factor (PDGF) have demonstrated to promote endothelial cell invasion *in vitro* creating fully formed blood-vessel-like structures (Howell et al., 2018).

As already cited above, other protein family that has been successfully delivered by means of polymeric hydrogels incorporating Laponite are bone morphogenetic proteins (BMPs). Laponite-BMP complexes embedded in HA hydrogels have been demonstrated to maintain the

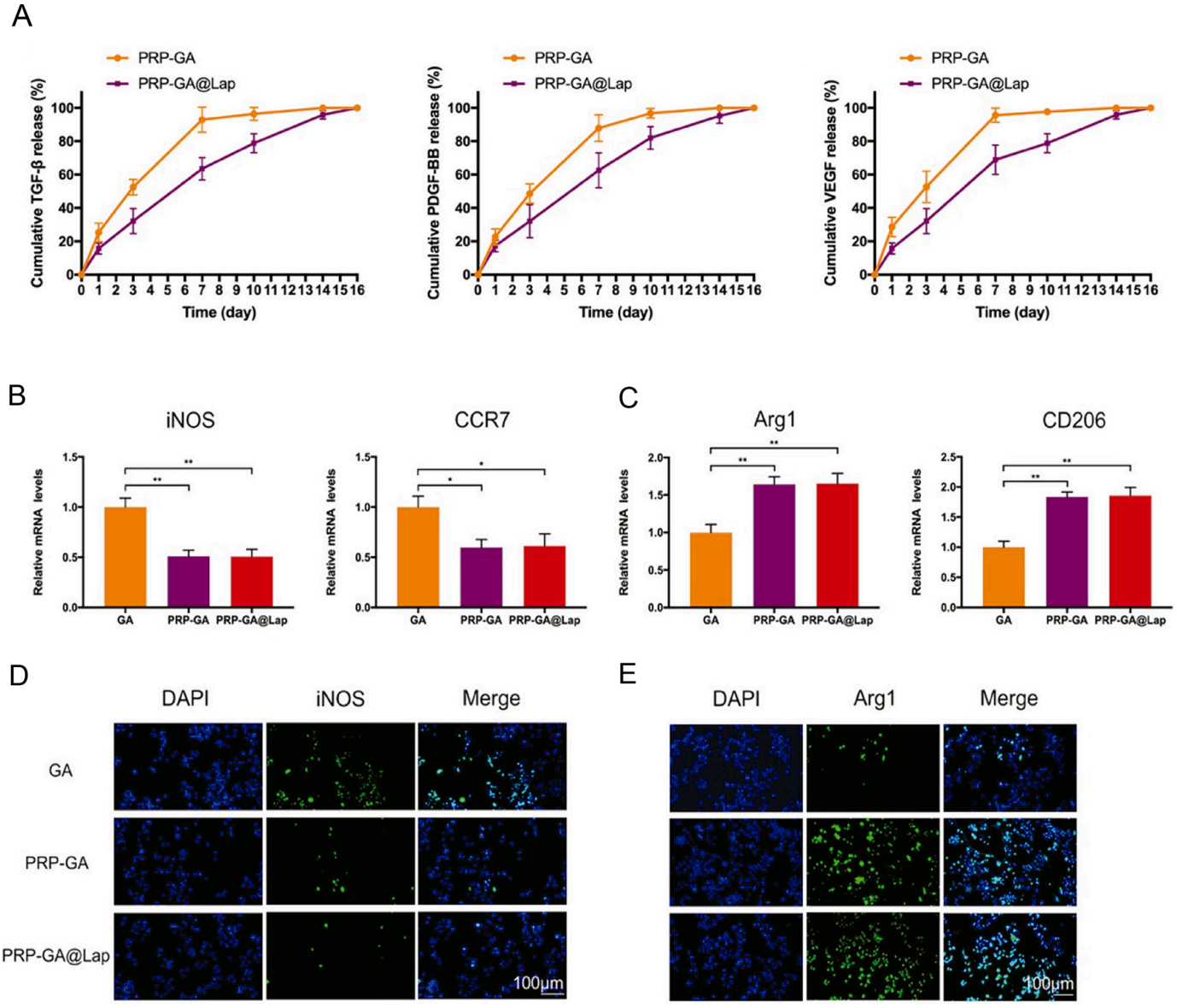

Fig. 4. PRP release from methacrylated gelatin – methacrylated alginate – Laponite hydrogels. (A) Growth factor release profiles of TGF-β, PDGF and VEGF of each group of hydrogels (n = 3/group). Effect of hydrogels on macrophage polarization *in vitro*: (B) results of qPCR of M1-related gene expression (CCR7 and iNOS) (n = 3/group); (C) results of qPCR of M2-related gene expression (CD206 and Arg1) (n = 3/group); (D) immunofluorescence staining of iNOS in RAW264.7 cells cultured on each group of hydrogels; (E) immunofluorescence staining of Arg1 in RAW264.7 cells cultured on each group of hydrogels. All experiments were replicated three times. *$p < 0.05$ and **$p < 0.01$. Reprinted with permission from Cao et al. (2023), content under the Creative Commons Attribution 4.0.

bioactivity of the protein and as a result, promote osteogenesis both, *in vitro* and *in vivo* (Zhang et al., 2020). The literature shows that such systems are able to sustain for over 6 weeks the delivery of BMP-2 *in vivo* and achieve ectopic bone induction at doses below the typical efficacy threshold reported (Kim et al., 2020). Some other works have taken a step ahead and fabricated bi-zonal alginate-methylcellulose-Laponite systems that can act as a depot of BMP-2 in one zone and TGF-β3 in the other, as a tool to direct cell differentiation towards the osteogenic and chondrogeneic lineages, respectively (Kilian et al., 2022). BMP-4 has also been loaded in Laponite-polymer hydrogels for wound healing, confirming the results obtained in the above-mentioned studies and controlling the release of the growth factor. In particular, HA-methacrylate-Laponite hydrogels were able to sustain the delivery of the protein, leading to a reduced collagen type I/III fraction and α Smooth Muscle Actin (α-SMA) production, which resulted in a decrease in scar formation (Chang et al., 2023).

Interestingly, Laponite-polymer nanocomposites have also been employed to sustain the delivery of products that contain multiple peptides and proteins. Such is the case of platelet-rich plasma (PRP) or the secretome derived from MSCs (Waters et al., 2016; Waters et al., 2018; Cao et al., 2023). The release of factors such as PDGF, TGF-β and VEGF present in PRP has been demonstrated to be sustained up to 2 weeks when loaded in methacrylated gelatin – methacrylated alginate – Laponite hydrogels. Moreover, *in vitro* studies employing such hydrogels enhanced the migration and proliferation of rat bone marrow MSCs and promoted angiogenesis and M2 macrophage polarization (Cao et al., 2023) (Fig. 4). On the other hand, the MSC-derived secretome comprises a plethora of paracrine biomolecules that these cells produce, including growths factors, cytokines and enzymes of proteic nature that present angiogenic, regenerative and anti-inflammatory properties (Munoz-Perez et al., 2021; Ceruso et al., 2021). Gelatin – methacrylate – Laponite nanocomposites have been employed for the sustained delivery of human bone marrow derived MSC secretome. Secretome analyses showed the presence of important angiogenic and regenerative factors including VEGF, BMP-2, FGF2 or angiogenin.

Importantly, the nanocomposite system was capable of decreasing the release rate of these factors over 15 days, which were proven to maintain their bioactivity promoting angiogenesis and cardioprotection *in vitro* (Waters et al., 2016). Laponite-polymer nanocomposites loaded with MSC-derived secretome have also demonstrated their therapeutic potential *in vivo*. Waters *et al.* incorporated human adipose MSC derived secretome in gelatin – Laponite hydrogels with the aim to obtain a sustained release of therapeutic proteins in the peri-infarcted myocardium of rats. The assessment of the harvested secretome showed that it was rich in factors such as VEGF, angiogenin, endostatin/collagen XVIII, hepatocyte growth factor (HGF), endothelin-1, pentraxin-related protein (PTX3) or metalloproteinase inhibitor 1 (TIMP-1). Up to 21 days after intramyocardial injection of the secretome-loaded nanocomposite hydrogels, increased in capillary density, reduced scar formation and improved cardiac function were observed (Waters et al., 2018).

4. Conclusions

Laponite has demonstrated a great potential for promoting a sustained delivery of protein therapeutics. These drug molecules can be loaded in the nanoclay not only at inter-layer sites, but also onto the surface and edge of the crystals and at inter-particle sites. The ionic nature of this nanoclay, together with its high surface area and its hydrophilicity enable interactions with a wide range of functional groups present in all amino acids. As a result, protein therapeutics can be successfully loaded in Laponite without altering their bioactivity, which significantly reduces their burst release and prolongs their delivery. This opens up the path towards reduced doses and administrations, diminishing side effects and importantly enhancing patient compliance.

However, to precisely control the loading and release of proteins many factors are to be considered – including the physicochemical properties and concentration of Laponite and the target protein, the pH and composition of the dispersion buffer, as well as the degradation of the nanoclay –. Despite encouraging results have been obtained for the delivery of protein therapeutics from plain Laponite formulations and, more remarkably, from Laponite-polymer nanocomposites, optimized studies thoroughly considering them all in an integrated fashion are yet to be conducted to fully harness the multiple benefits that Laponite offers. A better understanding of how protein-Laponite complexes are formed in terms of stability, stoichiometry and reversibility will also surely contribute. Moreover, it is still necessary to study in depth the effects that the physiological *in vivo* environment, rich in molecules that can also interact with Laponite, has over protein delivery.

The intense research that is being conducted over the last years makes the use of Laponite for drug delivery a hot topic and will undoubtedly lead to overcome these challenges shortly. The resolution of these issues, supported by the authorities already recognizing the biocompatibility of the nanoclay, will surely pave the way towards the design of Laponite-based protein delivery systems that can be adapted to the vast number of applications that therapeutic proteins present, including endocrine disruptions, tissue defects and immune diseases.

CRediT authorship contribution statement

Ainhoa Gonzalez-Pujana: Writing – original draft, Conceptualization. **Manoli Igartua:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization. **Rosa Maria Hernandez:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization. **Edorta Santos-Vizcaino:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization.

Declaration of competing interest

None.

Data availability

Data will be made available on request.

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