Alkaline Phosphatase: A Reliable Endogenous Partner for Drug Delivery and Diagnostics

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Since the 1960s the membrane-bound enzyme alkaline phosphatase (ALP) has been utilized in drug delivery. As it cleaves phosphate substructures from drugs, auxiliary agents, and even from the surface of nanocarriers, this enzyme enables the design of drug delivery systems that can alter their properties in the body on demand. Anionic nanocarriers exhibiting bioinert properties can alter their surface to interactive once having reached the target site as due to an ALP-triggered cleavage of anionic phosphate groups from their surface charge converts to cationic improving for instance cellular uptake. Moreover, features such as the accumulation of nanocarriers at the target site or a targeted drug release triggered by ALP can be introduced. In addition, ALP is utilized to improve the potential of numerous diagnostic systems. Within this review, one provides an overview about the activity, selectivity, and distribution of this enzyme, as well as the great variety of applications in drug delivery and diagnostics making use of it.

1. Introduction

Over the last decades, enzyme-responsive drug delivery systems (DDS) have been broadly investigated as a useful tool to improve drug solubility (i), to shuttle drugs to the diseased tissue (ii), to improve drug uptake (iii) and to provide a controlled and targeted drug release (iv). Among the various endogenous enzymes that have already been successfully employed for drug delivery like matrix metalloproteases, hyaluronidase, lipases, and trypsin, phosphatases are of particular interest. As these enzymes cleave phosphate esters being involved in many biological functions like information storage and transfer, energy exchange and membrane fluidity, they hold key roles in many physiological processes. Phosphatases are therefore widely distributed all over the body offering numerous target sites and abundant opportunities for the design of innovative dynamic DDS. Among phosphatases especially alkaline phosphatase (ALP, E.C.3.1.3.1) moved in the limelight of research as it cleaves phosphate esters including thioesters in a nonspecific but highly efficient manner. Already in the 1960s drug delivery experts started to utilize ALP in order to overcome delivery problems. By the design of phosphate ester prodrugs, the poor aqueous solubility of various drugs such as estramustine\(^{[1]}\) and prednisolone\(^{[2]}\) could be addressed. When given orally these prodrugs rapidly dissolve in gastrointestinal (GI) fluids, yet being efficiently absorbed from the intestinal mucosa. Their high membrane permeability is ascribed to the quantitative cleavage of the hydrophilic and polar phosphate substructure by intestinal membrane-bound ALP being located exactly there where high drug solubility has to be replaced by high membrane permeability. In the early 1970s first phosphate ester prodrugs came into clinical use. Since then ALP has been used to overcome many different drug delivery challenges such as i) the prevention of mucosal damage at the absorption site by masking the high local toxicity of drugs via temporary phosphorylation,\(^{[3]}\) ii) the polycation dilemma by generating charge converting nanocarriers (NCs) due to cleavage and release of anionic phosphate substructures on their surface at the target site,\(^{[4]}\) iii) prolonged mucosal residence times by making use of ionic interactions after sufficient mucus interpenetration,\(^{[5]}\) or iv) targeted drug release via a phosphatase-triggered disintegration/aggregation of NCs.\(^{[6,7]}\) Recently, the presence and role of ALP in various cancers and diseases have been in the spotlight leading to the development of new ALP detection techniques, disease treatment, as well as diagnostic approaches.\(^{[8-17]}\) Within this review, we aimed to provide an overview about ALP-responsive novel pharmaceutical excipients, drug delivery systems, and diagnostics by also giving a perspective about the distribution...
(Figure 1) and activity of ALP in the body as well as its specificity and mode of action. Accordingly, the potential of phosphorylated excipients for drug delivery and diagnostics is highlighted. Moreover, phosphate-bearing nanosystems such as liposomes, solid lipid nanoparticles (SLN), nanostructured lipid carriers, self-emulsifying drug delivery systems (SEDDS), and polymeric nanoparticles, whose efficacy can be strongly enhanced by an enzyme-triggered phosphate release, are discussed.

2. Alkaline Phosphatase

ALP is a large subclass in the hydrolase enzyme superfamily that catalyzes the hydrolysis of a wide variety of phosphate mono-, di-, triesters, inorganic pyrophosphates, phosphorami- dates, phosphate monoesters, sulfate monoesters, and sul- fonate monoesters. It is a membrane-bound glycosylated metalloenzyme that is one of the most ubiquitous enzymes in nature being found in plants, algae, bacteria, and all animals suggesting its involvements in fundamental biochemical processes. Since its discovery in the 1920s, many types of ALPs have been identified and characterized providing a better understanding of its evolution. In particular, ALP from Escherichia coli, a prototype for all ALPs, has been widely and thoroughly studied since the 1950s regarding its crystal structure and catalytic properties. In the 1980s, various mammalian ALP complementary DNAs had been cloned and the corresponding enzymes have been characterized. In the 1990s, many biological functions were proposed for ALP. In 2001, the 3-D structure of human placental ALP was discovered allowing deeper insights in the active site and critical residues of the enzyme, and providing valuable information for the design of enzyme inhibitors, prodrugs, and substrates for biochemical applications, ALP-related disease treatment, and drug delivery.

2.1. Distribution and Physiological Functions of ALP

For the design of ALP-related prodrugs, DDS, and diagnostics, a profound knowledge about the distribution, properties, and physiological functions of ALP is mandatory. In humans, there are four genes encoding four corresponding ALP isozymes. Three of them are tissue-specific ALP (TSAP), namely placental ALP (PLAP), germ cell ALP (GCAP), and intestinal ALP (IAP). The fourth is tissue nonspecific ALP (TNAP) representing the most abundant isozyme that is expressed mainly in bone, liver, kidney, and central nervous system. These isozymes can be distinguished from each other by certain parameters such as electrophoretic mobility, molecular weight, immunologic properties, heat stability, and effect of inhibitors.

As ALPs are membrane-bound glycoproteins attached to the exterior of the plasma membrane, they are highly expressed in the extracellular space and less expressive in cytoskeleton, mitochondria, peroxisomes, nuclei, endoplasm, or lysosomes; while minor expressions are shown in endosomes and the Golgi apparatus. On cultured Caco-2 cells, 88–90% of ALPs are distributed over the cell membrane, 3–7% are in cytosol and 3–6% are secreted. IAPs are expressed on the apical brush border membrane of enterocytes and secreted into the intestinal lumen by membrane shedding. They have a critical function in nutrient absorption and anti-inflammation. The appearance of IAP in serum is proven to be related to the ingestion of fat and coabsorption with triglycerides into the lymph before being released into the blood circulation. TNAPs are expressed in virtually all tissues. They are abundantly expressed on osteoblasts, whereas acid phosphatase is expressed on osteoclasts. Both of them play vital roles in skeletal mineralization and development. GCAP is present at the plasma membrane of migrating primordial germ cells and early-stage testicular germ cells;
Table 1. Distribution of ALP at various tissues in the body that can be potential routes or target sites for drug delivery.

| Tissue/organ | Isoform/isozymes | Known or proposed functions of the isozyme | ALP activity(\(^{a}\)) (IU g\(^{-1}\) tissue) | Refs. |
|--------------|------------------|--------------------------------------------|--------------------------------------------|-------|
| Intestine    | IAP              | - Regulation of intestinal surface pH      | 38 ± 14                                    | [43–47]|
|              |                  | - Absorption of lipids                     |                                            |       |
|              |                  | - Detoxification of free nucleotides and bacterial lipopolysaccharides (LPS) |                                            |       |
|              |                  | - Possible modulation of the gut microbiota |                                            |       |
|              |                  | - Regulation of transmucosal passage of bacteria |                                        |       |
|              |                  | - Attenuation of intestinal inflammation   |                                            |       |
|              |                  | - Dephosphorylation of extracellular adenosine triphosphate (ATP) to adenosine |                                            |       |
| Colon        | IAP, colonic TNAP| - Reduction of oxidative stress and inflammation | 2.3 ± 0.8                                  | [48,49]|
| Testis, malignant trophoblasts, testicular cancer | GCAP | - Sperm glycolytic reactions and fructose formation | 0.5 ± 0.1 (in testis) 33 ± 5 (in seminoma) | [33] |
| Placenta, various solid tumors (breast, lung, ovary, gastrointestinal tract, testicular cancer, etc.) | PLAP | - Tumor marker | 69 ± 44 (placenta) | [30,31,32] |
| Bone         | TNAP             | - Bone mineralization and development       |                                            | [31] |
| Liver        | TNAP             | - Indicator of osteoblastic activity         |                                            |       |
| Kidney       | TNAP             | - Hydrolisis of phosphorylcholine            | 2.6 ± 1.4                                  | [53] |
| Nasal cavity, airway surfaces | TNAP, PLAP | - Antiendotoxin mediator and anti-inflammatory action |                                        | [22,33,54] |
| Lung         | TNAP             | - High activity in lung fibrosis and smoking due to appearance of placental-like AP | 2.1 ± 0.5                                  | [40,55]|
| Vagina, cervical mucosa and endometrium | TNAP | - Indicator of estrogen action |                                            | [54,57] |
| Eyes         | TNAP             | - Specific function is unknown               |                                            | [58,59]|

\(^{a}\) Data for ALP activity are referred from ref. [47], expressed as mean ± SEM.

while PLAP is synthesized in the syncytiotrophoblast after the twelfth week of pregnancy and is secreted into the maternal circulation.\(^{31}\) Various cancers release PLAP or GCAP into the circulation. PLAP is extensively studied because of its ectopic expression in tumors.\(^{31}\)

ALPs are also present in human serum and used as biomarkers for the diagnostic of liver diseases, bone disorders, and cancers.\(^{31,36,37}\) The majority of ALP in serum (>80%) is released from liver and bone, and just a few percent is from the intestine.\(^{38}\) The mechanism for ALP liberation from cell surfaces is not clear and may differ from organ to organ. It could involve detergent action due to high concentration of bile salts in the liver, tissue phospholipase activation, the action of proteases, or some unique surfactant-like particles in the intestine.\(^{39}\) The presence of significantly increased serum level of PLAP or GCAP in healthy and nonpregnant subjects can be a reliable indicator of an underlying malignant tumor. Most tumors are known to express more than one ALP isozyme, e.g., ovarian cancers show co-expression of PLAP and GCAP.\(^{31,40}\) Besides being employed as tumor markers in blood test, ALPs at tumor could also be used as i) membrane targets for tumor immunolocalization\(^{31}\); or ii) potential trigger factors to activate the site-specific release of drugs or fluorescence probes in tumor imaging.\(^{41}\)

Table 1 provides the ALP isoforms and their functions at the corresponding tissues that can be potential routes or target sites for drug delivery. In the intestine, ALPs cleave lipopolysaccharides (LPS, Gram-negative bacteria endotoxin) and extracellular nucleotides leaking from damaged and ischemic cells as physiological substrates. As dephosphorylation of LPS lowers the activation of toll-like receptor 4 (TLR-4), ALP can alleviate or prevent the local and systemic inflammatory response in patients with sepsis or severe ulcerative colitis.\(^{42,43}\) In bone, tooth, and cartilage, ALP plays vital roles in mineralization, dephosphorylation of extracellular pyridoxal 5’-phosphate, and thus vitamin B6.
metrical subunits with pI 6.0 and sidechains.[38] The membrane-bound form of ALP is probably a similar acid amine sequence, yet different carbohydrate and lipid metabolism.[29] Furthermore, ALP may be involved in regulating DNA synthesis and intracellular protein synthesis.[34]

2.2. Enzyme Structure and Active Site

Structurally, ALP is a dimer of two identical or non-identical subunits linked by a disulfide bridge. For example, fetal IAP contains two different subunits, one with isoelectric point (pI) 6.0 and molecular weight (Mw) ≈ 64,000, and the latter has pI 5.5 and Mw ≈ 72,000; while adult IAP consists of two identical subunits with pI 5.9 and Mw ≈ 70,000; or PLAP contains also two identical subunits with pI 6.0 and Mw ≈ 64,000.[60] All TNAP have similar acid amine sequence, yet different carbohydrate and lipid side chains.[38] The membrane-bound form of ALP is probably a tetramer.[15] The dimension of the enzyme molecule is approximately 100 × 50 × 50 Å, and the distance between two active sites is approximately 30 Å.[61] ALP is a glycoprotein attaching to the cell membrane by a glycan–phosphatidyl–inositol anchor. The carbohydrate side chains of ALPs are terminated by sialic acid, except for IAP.[62] Removal of carbohydrate units does not deteriorate ALP catalytic activity.[18] However, posttranslational changes in glycosylation may alter catalytic properties.[63] Although ALP is a comparatively large membrane-bound enzyme, phosphorylated compounds can easily access its active sites. Even phosphate substructures on the backbone of polymers and on the surface of different types of NCs were shown to be efficiently cleaved by ALPs.[64–69] This observation is in line with the dephosphorylation of endogenous polymers such as LPS, phosphoproteins, or DNA by ALPs.[65,70–72]

Although sequence comparisons demonstrate just 25%–30% homology amongst the E. coli ALP and the yeast and mammalian ALPs, the active center of ALP is greatly conserved explaining their common catalytic property, i.e., hydrolysis of phosphomonoesters into inorganic phosphate and alcohol. The essential components for catalytic activity include the catalytic Ser102, the three metal ion sites: Zn1 (occupied by the first Zn2⁺), Zn2 (occupied by the second Zn2⁺), and Mg (occupied by Mg2⁺) as well as their ligands (Figure 2) that are necessary for the enzymatic activity.[22] The catalytic mechanism involves the activation/deprotonation of the hydroxyl group of Ser102 residue by Zn2 and Mg inducing a nucleophilic attack on the phosphorus center of the phosphate ester (−O)−P−O−R and the preparing for the formation of a covalent serine-phosphate intermediate. The enzyme–substrate complex is completely formed by the electrostatic, hydrogen bond, and dipole–dipole interactions between Zn1, Zn2, Ser102, Arg166, and the three nonbridging oxygen atoms (−O)−P− of the substrate, while the bridging oxygen atom P−O−R of the substrate is coordinated with Zn1. Zn1 plays an important role in promoting the removal of the −O−R leaving group. The formation of the covalent Ser102-P intermediate leads to the inversion of the phosphorus center and the release of the leaving group. Afterwards, a nucleophilic hydroxide ion −OH coordinated to Zn1 attacks the phosphorus atom and hydrolyzes the covalent Ser102-P intermediate to form the noncovalent enzyme-phosphate E-Pi complex and restore the nucleophilic Ser102. The release of inorganic phosphate from the E-Pi complex to restore free enzyme can be facilitated by the reproteination of Ser102 and the increased mobility of the Arg166 side chain.[19,73] The Mg-binding site appears to play no direct role in the catalysis steps, its role is likely to provide the necessary environment for the catalytic steps and to stabilize the enzyme structure in the catalytically most active form.[74] The substitution of Zn²⁺ with Mg²⁺ does not completely destroy the hydrolytic activity, meanwhile increases the transferase activity of the enzyme.[75] Minor substitutions or mutation at several positions in the active site can lead to small or dramatic changes in their catalytic properties.[70,74] For example, active site residues Asp-153 and Lys-328 in E. coli ALP are changed with histidines in mammalian ALP being responsible for more alkaline optimum pH and less thermal stability and magnesium activation of mammalian ALPs.[74]

ALP active site pocket is very spacious, of ≈10 × 20 Å² wide and ≈15 Å deep.[19] Because of its shallow active site, ALP binds just
the phosphate part of the substrate. Nonetheless, the remaining portion of the substrate can have a major impact. Akkus et al. investigated the enzymatic cleavage of various phosphorylated surfactants by IAP demonstrating pronounced differences dependent on the type of surfactant as illustrated in Figure 3. Generally, a more hydrophilic substructure next to the phosphate group seems to facilitate its hydrolytic cleavage. Polyphosphates such as tripolyphosphate or Graham's salt are, for instance, rapidly cleaved by IAP, whereas the phosphate group of cetylphosphate is only to a minor extent if at all cleaved by the same enzyme.

The shallow active site of ALP, however, makes it difficult to design selective and high-affinity inhibitors. Discovery of potent and selective ALP inhibitors could help to better understand the role of ALPs in fundamental biological processes and unveil the secrets of life. As the enzyme active site possesses a high density of positive charges, the substrate electric charge, electron-withdrawing capability of the leaving group, pH, and pK_a of the leaving group play important roles in binding efficacy and the overall catalytic/inhibitory activity. It is evidenced that the diaionic form of phosphate monoester is the favored substrate for ALP, and that diaionic form of the free phosphate P_i binds to the enzyme active site as strong or stronger than the trianion P_i. Burlington et al. investigated the impact of electrostatic interaction on binding of inhibitors to ALP. They found that N-sulfonylphosphoramidates were stronger bound to ALP than N-phenylphosphoramidates suggesting that the negatively charged sulfonyl group played some role in interacting with metal atoms in the enzyme active site. This theory is also supported by the efficient cleavage of polyphosphates by ALP. Trianionic N-sulfonylphosphoramidates did not show a better enzyme binding than diaionic N-sulfonylphosphoramidates. Moreover, ground-state charges of nonbridging oxygen atoms in phosphate or sulfate group were shown to be an ultrasensitive factor to the catalytic activity of ALP. The higher absolute value of this charge results in higher catalytic proficiency.

2.3. ALP Activity

As ALP activity directly affects the performance of prodrugs as well as phosphate-functionalized DDS and diagnostics, a detailed knowledge about ALP activity at the target organ or tissue is essential. In Table 1, an overview on the activity of ALP isotypes in various tissues is provided. The activity is listed on each tissue in international unit (IU) being defined as the amount of enzyme that catalyzes the transformation of one micromole of substrate per minute under specified conditions. A direct comparison has nonetheless to be done cautiously, as different substrates are used, and test conditions may vary from tissue to tissue. Overall, ALP activity also changes depending on age, sex, hormonal status, medical condition, and diet. In children, ALP activity associates with height and weight, and until puberty the bone ALP accounts for 77%–87% of the total ALP activity. Bone ALP activity is also reported to increase during the first two trimesters of pregnancy, and in the last trimester PLAP activity contributes mainly to the overall ALP activity. Bone ALP activity is almost equal to liver ALP activity in healthy adults. Patients with hypophosphatasia, an inherited metabolic disorder characterized by defective bone mineralization have low TNAP activity but normal activities from other ALP isozymes. In patients with leukemia, ALP from chronic granulocytic leukemia leukocytes has lower specific activity than ALP from normal leukocytes, while ALP from reactive granulocytic leukemia leukocytes has a very high specific activity. IAP is present primarily at the intestinal mucosa and its activity increases after eating, especially high-fat meals. There are reports claiming that catalytic properties of free ALPs are different from membrane-bound ALPs, and that ALPs in circulation seem to be metabolically inactive. In some other reports, activities of IAP in free and associated states showed no drastic difference. It has been shown that long lipid chains can inhibit extracted IAP and skeletal ALP, but much less effectively inhibit the same isozyme secreted in serum, suggesting the difference in the structure of membrane-bound and free version of the same enzyme at some lipophilic regions.

ALP is expressed on many cell lines that are commonly used in vitro studies. The enzyme expression on cell surface and its activity is highly dependent on the components of culture medium and the culture day. Caco-2 cells widely used in drug/NC absorption, transportation, and disposition studies, can differentiate under specific culture conditions forming an epithelial monolayer resembling human small intestinal mucosa. Caco-2 cells express just half of the ALP activity of human intestinal mucosa. ALP activity of Caco-2 cells cultured for 15 d in FBS supplemented modified Eagle’s medium was found to be 830 ± 250 mIU mg^{-1} protein. HEK 293 cells, a cell line widely used in gene transfection and cancer research, express ALP on their surface and ALP activity is 15 ± 3 mIU mg^{-1} protein. Addition of oxidants such as H_2O_2, monochloramine, or tert-butyl hydroperoxide to culture medium was shown to increase ALP activity in Caco-2, HT29, and IEC18 cells. ALPs hydrolize not only phosphomonoesters but also S-phosphorothioates, phosphomonoesters, and thio phosphates. It can catalyze sulfate monoester and phosphonate monoester hydrolysis reaction but with a very low preference compared to phosphate monoester. ALPs are also shown to have phosphodiesterase and transferase properties. Besides physiological substrates such as lipopolysaccharides, nucleotides, glucose-6-phosphate, phosphorylcholine, circulating pyrophosphate, phosphoethanolamine (PEA) or pyridoxal 5′-phosphate (PLP), a vast variety of phosphate compounds can be substrates of ALP such as glycerophosphate, polyphosphates, bis p-nitrophenyl phosphate, methyl p-nitrophenyl phosphate, phosphatidates etc. These compounds can be utilized as building blocks or structural analogs to design new ALP responsive excipients.

ALP catalytic activity is dependent on pH, substrate concentration, temperature, and buffer components. As suggested in its name, ALP performs optimally at pH 8–10. However, this is not always the case. The term was coined in the 1930s to rather distinguish it from acid phosphatase. ALPs have their maximal activity at alkaline pH only at high substrate concentrations as in clinical and research laboratories. At physiological substrate levels in nanomolar or micromolar range, the pH optimum is close to the physiological pH value of 7.4. Practically, the enzyme...
Figure 3. Enzymatic phosphate cleavage by IAP on various phosphorylated surfactants showing lower phosphate release from surfactants with long lipid chains, i.e., dipalmitoyl-sn-glycero-3-phosphatidic acid (PA) and 2-((2,3-bis(oleoyloxy)propyl)dimethylammonio)ethyl hydrogen phosphate (DOCP). Adapted with permission.[81] Copyright 2021, Elsevier.

1,2-Dipalmitoyl-sn-glycero-3-phosphatidic acid disodium salt (PA)

Polyoxyethylene (9) nonylphenol monophosphate ester (PNPP)

2-((2,3-Bis(oleoyloxy)propyl)dimethylammonio)ethyl hydrogen phosphate (DOCP)

C12-15 alcohol 3 ethoxylate phosphate ester (PME)

Dioctanoyl glycerol pyrophosphate (DGPP)
is still active even at pH 5.5, of course with lower performance.\textsuperscript{[18]} Enzymatic reaction can be quenched by adjusting reaction pH to a very acidic value.

In vitro studies, ionic strength and buffering agents can affect hydrolysis reaction kinetics. The presence of chloride ion Cl\textsuperscript{−} at 1 M can enhance the dissociation rate of phosphate from E-Pi complex 15 times.\textsuperscript{[18]} Free IAP is remarkably activated by high ionic strength at pH 8.0 while the membrane-bound enzyme is slightly inhibited under the same conditions.\textsuperscript{[85]} In the presence of buffering agents and phosphate acceptors such as ethanolamine or Tris, ALP can catalyze a transferase reaction with the phosphate transferring to the alcohol group forming another phosphate monoester.\textsuperscript{[97]} ALP activity was shown to be poor in glycine and 2-amino-2-methyl-1-propanol buffers, 15–70\% catalytic activity could be lost after incubation for 25 min at 37 °C due to the enzyme instability; whereas most of its activity remained in diethanolamine and 2-amino-2-methyl-1,3-propandiol buffers under the same condition.\textsuperscript{[98,99]} It has to be noticed that 2-amino-2-methyl-1-propanol is used as a buffer in the common method for ALP assay recommended by International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) in 1983 at the recommended temperature 30 °C.\textsuperscript{[100]}

In the temperature range of 25–37 °C, \( K_m \) of the hydrolysis reaction of p-nitrophenyl phosphate (pNPP) by human ALP decreases with increased temperature.\textsuperscript{[101]} The optimum pH of ALP is slightly influenced by temperature, which is less than 0.03 pH unit per degree Celsius.\textsuperscript{[102]} Different isozymes have different sensitivity to heat depending on their carbohydrate side chains.\textsuperscript{[48]} Besides electrophoresis, this property is used to distinguish each ALP isotype. Thermostable PLAP is still active after being heated to 65 °C for 1 h, while TNAP is thermostable and is inactivated under such conditions. IAP is shown to be more thermostable than TNAP and still functions when treated at 56 °C for more than 1 h; however it is unstable at 65 °C.\textsuperscript{[32,35]} It has been shown that PLAP activity increased by 20–30\% during heat treatment.\textsuperscript{[40]}

### 2.4. Inhibitors, Activators, and Regulating Factors of ALP

Phosphatase inhibitors are often used in various in vitro studies especially on cultured cell models and on freshly excised tissue to evaluate the impact of ALP on the performance of phosphate functionalized DDS.\textsuperscript{[7,81,103,104]} Performing cellular uptake studies of phosphorylated NCs with and without inhibition of ALP, for instance, can show the effect of this enzyme on the performance of these delivery systems as depicted in Figure 4. Table 2 lists some common inhibitors and activators of various isotypes of ALP as well as their proposed mechanism.

Common inhibitors like vanadate, pervanadate, or okadaic acid are present in some commercial inhibitor cocktails which are originally used to stabilize protein during protein extraction from cells. However, they were shown to affect cell viability, functionalization, growth, and cellular uptake as they may induce changes in the redox state of mitochondria.\textsuperscript{[106,107]} These effects are dependent on the inhibitor concentration, and incubation time. Therefore, type and concentration of phosphatase inhibitors used in experiments on cells should be taken into consideration.

ALP is also competitively and reversibly inhibited by inorganic phosphate, the product of dephosphorylation reaction. Furthermore, l-cysteine, l-tryptophan, l-phenylalanine, and l-leucine were found to inhibit human TSAP,\textsuperscript{[31,108]} while l-homoarginine was shown to be a TNAP inhibitor.\textsuperscript{[109,110]} The mechanism involved the interaction of Arg residue and Zn1 in the ALP active site with l-amino acids through their carboxylic and amine groups, respectively, and thus interfering the hydrolysis of phosphate-ALP intermediate.\textsuperscript{[31]} ALP is irreversibly inhibited if there is a permanent conformational change at its active site or its structure is deformed. As a metalloenzyme, ALP is inhibited by metal ion chelators such as EDTA or polysaccharides. The inhibition is irreversible by removing the zinc from the active site. As a protein, 3D structure of ALP is a decisive factor impacting its activity. Because disulfide bonds are responsible for the tertiary structures of proteins, any chemical or physical modification to these —SH groups can induce changes in protein structure.

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**Figure 4.** Confocal laser scanning images showing the effect of ALP inhibitors on cellular uptake of phosphate-functionalized NCs. Blue objects depict cell nuclei, yellow dots are NCs. With the presence of ALP inhibitors, NCs were internalized at much lower level compared to the cellular uptake in the absence of ALP inhibitors. A) Live imaging and B) cross-section view of Caco-2 cell monolayer incubated with 0.005\%(v/v) lumogen yellow loaded nanocarrier formulations: control nanocarriers (1), PLL-SA/TPP decorated nanocarriers (2), PLL-SA/TPP decorated nanocarriers co-incubated with PIC2 (3), and PLL-SA/PA decorated nanocarriers (4). PLL-SA/TPP: tripolyphosphate coated PLL-SA decorated nanocarriers. PLL-SA/PA: phytic acid coated PLL-SA decorated nanocarriers. PIC2: phosphatase inhibitors cocktail 2 from Sigma Aldrich. Adapted with permission.\textsuperscript{[105]} Copyright 2021, Elsevier.
Table 2. Inhibitors and activators of ALP.

| Type of ALP | Mechanism | Inhibiting/activating concentration | Refs. |
|-------------|-----------|------------------------------------|-------|
| **Inhibitors** | | | |
| Inorganic phosphate | ALP | Competitive inhibition, reversible occupancy of the enzyme active site | $K_{i}^{a)} \approx 1.8 \times 10^{-3}$ m for IAP | [22,78,137] |
| | | | $K_{i} \approx 5 \times 10^{-4}$ m for E. coli ALP | |
| | | | At 1 $\times 10^{-3}$ m inhibits =25% ALP activity, at 10 $\times 10^{-3}$ m inhibits =80% activity | |
| Molybdate | ALP | Competitive inhibition | | [138,139] |
| Vanadate | ALP | Similar structure to phosphate, competitive and reversible inhibitor | $K_{i} \approx 5 \times 10^{-6}$ m for E. coli ALP; 2.1–3.5 $\times 10^{-6}$ m for calf IAP | [106,123,140] |
| Arsenate | ALP | Reversible and competitive inhibitor | | [123,139] |
| Theophylline | IAP | Reversible, noncompetitive inhibitor | $K_{i} 102 \pm 4 \times 10^{-6}$ m | [123] |
| Aminophylline | ALP | IC$_{50}$ 0.08–0.2 $\times 10^{-3}$ m | [141] |
| L-Cysteine | ALP | $K_{m}^{b)}$ for p-nitrophenylphosphate is 0.39 $\times 10^{-3}$ m | 0.5 $\times 10^{-3}$ m | [108,141] |
| | | IC$_{50}$ 0.043 $\times 10^{-3}$ m | | |
| L-Phenylalanine | IAP and PLAP | Uncompetitive inhibition | | [32,60,109] |
| L-Leucine | GCAP, IAP, and PLAP | Uncompetitive inhibition | | [51,32] |
| | | GCAP is 10–20 fold stronger inhibited than PLAP | 5 $\times 10^{-3}$ m | |
| L-Leu-Gly-Gly | PLAP | Uncompetitive inhibition | | [52] |
| L-Phe-Gly-Gly | PLAP | Uncompetitive inhibition | | [32,60] |
| Levamisole | All ALP except IAP and PLAP | Stereospecific inhibition | | [60,117,143] |
| | | - $K_{i} 1.8 \times 10^{-6}$ m, IC$_{50}$ 19.2 $\times 10^{-6}$ m for bovine TNAP | |
| | | - 10 $\times 10^{-3}$ m inhibits more than 80% activities of most ALPs | |
| 2,5-dimethoxy-N-(quinolin-3-yl)benzenesulfonamide | TNAP | Uncompetitive inhibition. Strongly inhibit TNAP, while slightly inhibit IAP and PLAP | | [114] |
| Imidazole | TNAP | Competitive binding to Mg binding site. Reversible in the presence of Mg$_{2+}$ | | [146] |
| Divalent cations Zn$_{2+}$ | All ALP | Competitive inhibition against TNAP | | [145] |
| Excess Mg$_{2+}$ | Calf IAP | Pyrophosphatase and ATPase activities are strongly inhibited | | [93] |
| Urea | Bone IAP, kidney ALP | May rupture hydrogen bonds leading to unfolding of polypeptide chains and dampening enzymatic activity | IC$_{50}$ = 1.5 m | [109,346] |
| Coumarin sulfonate | IAP, TNAP | Binding of sulfonamide group inside ALP active site | $K_{i} 0.05–25 \times 10^{-6}$ m | [143] |
| Sulfonylurea derivatives | Bovine IAP, TNAP | Competitive inhibition against TNAP | | |
| | | Noncompetitive inhibition against IAP | | |
| Thiazole derivatives | IAP, TNAP | Interaction with Zn$_{2+}$ ions, formation of hydrogen bonds with Ser, His, Arg and Asp residues | | [142] |
| Sulfadiazinyl acyl/arylthiourea derivatives | IAP | Formation of hydrogen bonds with His, Arg, Asp residues | IC$_{50}$ 0.251 $\pm$ 0.012 $\times 10^{-6}$ m | [148] |
| Okadaic acid—a polyether fatty acid | ALP, protein phosphatase | Hydrophobic interaction between okadaic acid and specific regions of enzyme | 100 $\times 10^{-6}$ m | [106] |
| Fluoride | Pea plant ALP | Hydrophobic interaction with the enzyme’s substrate binding site | 1–10 $\times 10^{-3}$ m | [26] |

(Continued)
Table 2. (Continued).

| Type of ALP | Mechanism | Inhibiting/activating concentration | Refs. |
|-------------|-----------|-------------------------------------|-------|
| Chelating agents (EDTA, EGTA, polysaccharides, polynucleic acids ...) | ALP | Capture metal ions, irreversible inhibitor | EDTA concentration ≥ 1 × 10⁻³ M | [24,123,141] |
| Sulphydrylic reagents (dithiothreitol (DTT), 2-mercaptoethanol,...) | ALP | Possible dissociation of ALP dimer | 1 × 10⁻³ M DTT inhibits >98% activity | |
| Hg²⁺ | ALP | High affinity to —SH groups in ALP molecule | | [24] |
| Protein denaturants (guanidine HCl, urea, sodium dodecyl sulfate, etc.) | ALP | Interacting with and disturbing hydrogen-bonding and/or hydrophobic bonding in enzyme structure | | [149] |
| Oxygen free radicals (Fe²⁺/ascorbate, etc.) | Rat IAP | Direct oxidation | | [150] |
| Methylxanthines, amrinone, amiloride | TNAP | Might be due to the irreversible oxidation of thiol groups | 20 × 10⁻⁶ M (95%) | |
| Pervanadate | ALP | —SH inhibitor, weak inhibition | 100 × 10⁻⁶ M (80%) | [106,151] |
| Phospholipids (cephalins, lecithins, and phosphatidylinositol) | Bone ALP | Competitive inhibition | Cephalin: Kᵢ 0.3 × 10⁻⁶ M at pH 9.5 | [86] |
| Activators | | | |
| Mg²⁺ | Mammalian ALP | - Binding of the metal with free ALP leading to a conformational change | 2–14 times increase in activity | |
| L-Cysteine | Liver ALP, PLAP | At 0.1 mmol L⁻¹: activate liver ALP 4–5-fold, and PLAP 2–3-fold at pH 7.5–8.0 | 0.1 mmol L⁻¹ | [55] |
| Calyculin A | Bovine IAP | Increase ALP serine and threonine phosphatase activity | | [140] |
| Okadaic acid | Bovine IAP | Increase ALP serine and threonine phosphatase activity | | [140] |
| Ca²⁺ | cartilage ALP | ALP of Pyrococcus abyssi | | [52] |
| Zn²⁺, Mg²⁺, Mn²⁺ and Co²⁺ | ALP of Pyrococcus abyssi | | | [24] |
| EDTA (contrary results) | IAP, PLAP | Unknown | 20–400 × 10⁻⁶ M | [122] |
| Staurosporine | Bovine IAP | Enhance ALP serine and threonine phosphatase activities | 0.1 × 10⁻⁵ M | [145] |
| Sodium butyrate | ALP on HT-29 cells | Increase enzyme activity 1000 times | | [153] |
| Long PEG chain | Bovine IAP | Binding of PEG chain to certain sites of ALP | | [154] |

a) Kᵢ, inhibitor constant is the concentration required to produce half maximum inhibition. A small Kᵢ means that the inhibitor is bound tightly to the enzyme; b) K_m, Michaelis constant is the concentration of substrates when the reaction reaches half of V_max. A small K_m indicates high affinity with substrate. c) IC₅₀, half-maximal inhibitory concentration is the inhibitor concentration required to inhibit a biological process by half.

Protein tyrosine phosphatase (PTP), for example, contains —SH groups of cysteine in the catalytic site and therefore is inhibited by thiol-oxidizing agents. Unlike PTP, ALP does not contain —SH in its active sites. Nonetheless, it is also sensitive to common sulphydryl reagents such as 2-mercaptoethanol or dithiothreitol. These reagents may dissociate the dimer structure of ALP by disjoining the disulfide bridge and hence inactivate the enzyme. [24,111] Moreover, non-specific enzyme inhibitors such as protein denaturants or Hg²⁺ can impair enzyme structure and inactivate it.

Inhibitors of ALP showed great potential as i) drugs for the treatment of diseases related to pyrophosphate metabolism disorders [112] chronic kidney diseases, [113] renovascular hypertension, [114] or cancer; [115] ii) probes for fluorescence imaging of ALP in live cells or tissues. [116] ALP inhibitors such as (+)-p-bromotetramisole, 3-isobutyl-1-methylxanthine, theophylline, and vanadate were shown to activate cystic fibrosis transmembrane conductance regulator (CFTR) ion channel and could be used to increase the efficacy of cystic fibrosis (CF) therapy. [103] Levamisole has been shown to provide significant
Figure 5. Conversion of a phosphate prodrug (prednisolone phosphate) to its parent drug (prednisolone) by alkaline phosphatase.

Protection from colonic inflammation on rats. Moreover, phosphatase inhibitors targeting ALP can also be employed in treatment of many diseases such as inflammatory bowel disease, vascular smooth muscle cell calcification, and neuroimmune disorders.

Enzyme activators are usually low molecular weight molecules that bind to a site aside from the active site and increase enzyme activity. Some metal ions like Mg$^{2+}$, Mn$^{2+}$, Co$^{2+}$, and Ni$^{2+}$ can bind to ALP, and activate ALP via a conformational change. EDTA, an established ALP inhibitor, was reported to activate IAP at a concentration of $20-400 \times 10^{-6}$ m, but the mechanism was unknown. Selective activation or inhibition of ALP isozymes not only provides a deeper knowledge for disease treatment but also elucidates the exact role of each isozyme in metabolism and pathogenesis.

On the one hand, ALP can be downregulated by factors like but not limited to inflammatory cytokines, colonic inflammation (for IAP), dopamine, or prolonged use of diclofenac. On the other hand, the expression of ALP on cell surface has been shown to be upregulated by phosphate nonrelated factors such as all-trans-retinoic acid, vitamin K1 and K2, bovine serum albumin, dexamethasone, tumor necrosis factor-\(\alpha\), oncostatin M, dietary calcium, fat intake, colonic inflammation (for TNAP), or endoplasmic reticulum stress. In a research about the effects of potato starch ingestion on ALP activity in the small intestine in rats, authors showed that esterified phosphorus bound to the glucose molecule in potato starch might induce small increases in ALP activity in the small intestine of growing rats. Although the authors concluded that it was not possible to extrapolate this result directly to other animals and humans, the safety of phosphorylated nanosystems in terms of stimulating ALP activity should be considered when designing and testing novel ALP responsive DDS or diagnostic probes. In contrast to this observation, it is evidenced that low concentration of phosphate substrates (less than $30 \times 10^{-9}$ m) in cell surrounding environment can lead to an increased ALP activity. Farley showed that inorganic phosphate increased the stability of skeletal ALP activity but did not increase the level of ALP.

### 3. Phosphate Prodrugs

Prodrug strategies were utilized in the pharmaceutical field for over a century in order to improve the pharmacokinetics and bioavailability of active compounds. As a subcategory, phosphate prodrugs constitute a large number and in general, they increase solubility profile of the parent drug decreasing the usage of excipients in formulations and therefore reducing pill burden. ALP plays a key role in the transformation of phosphate prodrugs to their active form upon hydrolysis. In detail, phosphate modification introduces an ionized substructure providing high drug solubility in aqueous media. As illustrated in Figure 5, ALP enables a transition from ionized prodrug to nonionized parent drug ensuring high membrane permeability and thus increased absorption at the target site.

Phosphate monoesters constitute the largest group among phosphate prodrugs that are cleaved by ALP. They have been widely used via mostly parenteral but also oral routes for the treatment of various diseases including cancer, HIV infections, allergy, epilepsy, and so on as shown in Table 3. In general, these prodrugs are deriving from hydroxyl or amine groups of parent drugs consisting of a phosphate group directly or with a spacer arm attached to the main structure affecting enzyme interactions. Usage of spacer links has been benefited in or-
Table 3. Classification and current status of phosphate ester prodrugs activated by ALP.

| Prodrug            | Parent drug | Application route | Therapeutic target        | Chemical structure of prodrug | Clinical status | Trade name | Refs. |
|--------------------|-------------|-------------------|---------------------------|-------------------------------|-----------------|------------|-------|
| Fosamprenavir      | Amprenavir  | Oral              | HIV infections            | ![Chemical structure](image)   | Marketed        | Lexiva, Telzir | [169] |
| Fludarabine phosphate | Fludarabine | IV                | Leukemia, lymphoma        | ![Chemical structure](image)   | Marketed        | Fludara     | [158, 170, 171] |
| Estramustine phosphate | Estramustine | Oral, IV          | Prostate cancer           | ![Chemical structure](image)   | Marketed        | Emcyt, Estracyt | [1]   |

(Continued)
| Prodrug       | Parent drug | Application route | Therapeutic target          | Chemical structure of prodrug | Clinical status | Trade name       | Refs.          |
|--------------|-------------|-------------------|-----------------------------|-------------------------------|----------------|------------------|----------------|
| Polyestradiol phosphate | Estradiol | IM                | Prostate cancer             | ![Chemical structure 1](image1) | Marketed        | Estradurin       | [162]          |
| Prednisolone phosphate | Prednisolone | Oral | Inflammation, allergy | ![Chemical structure 2](image2) | Marketed        | Orapred ODT      | [158, 172]     |
| Fosfluconazole | Fluconazole | IV                | Fungal Infections           | ![Chemical structure 3](image3) | Marketed        | Prodif, Selfleks Flukosel | [158, 173] |
| Fosphenytoin | Phenytoin   | IV, IM             | Epilepsy                    | ![Chemical structure 4](image4) | Marketed        | Prodilatin, Proepanutin, Cerebryx | [158, 174, 175] |

(Continued)
Table 3. (Continued).

| Prodrug       | Parent drug | Application route | Therapeutic target                        | Chemical structure of prodrug | Clinical status | Trade name     | Refs.   |
|---------------|-------------|-------------------|------------------------------------------|-------------------------------|-----------------|----------------|---------|
| Fospropofol   | Propofol    | IV                | Anesthesia, sedation                     |                               | Marketed        | Lusedra        | [175]   |
| Fostamatinib  | Tamatinib   | Oral              | Chronic immune thrombocytopenia          |                               | Marketed        | Tavalisse      | [176]   |
| Etoposide phosphate | Etoposide | IV                | Cancer                                   |                               | Marketed        | Etopophos      | [167]   |
| TP-1287       | Alvocidib   | Oral              | Solid tumors                             |                               | Phase I (ongoing) | -             | [177]   |
| Amifostine    | WR-1065<sup>80</sup> | IV, SC           | Cytoprotective against chemotherapy and radiotherapy |                               | Marketed        | Ethyol         | [164]   |

<sup>80</sup> 2-((aminopropyl) amino)ethanethiol.
der to overcome the steric hindrance of the target phosphate group and therefore to achieve a desired enzymatic cleavage. Accordingly, Yuan et al. have compared the bioconversion rates of fosphenytoin and fosfluconazole in different in vitro set-ups including rat intestinal mucosa scraps, Caco-2 and Madin-Darby canine kidney (MDCK) cells which express ALP. Their study revealed that in all cases bioconversion of fosphenytoin to its parent drug was faster than that of fosfluconazole. This observation can be explained by the attachment of phosphoryl group to phenytoin by a spacer linker namely hydroxymethyl group. On the contrary, in the case of fosfluconazole, phosphate group was directly attached to tertiary carbon leading to a slower transition to its parent drug by ALP.\textsuperscript{[157]} Similar results were obtained by Kearney and Stella, indicating that phosphate monoester prodrug deriving from primary alcohol namely phosphoryloxy methyl phenytoin had a higher rate of dephosphorylation by human PLAP compared with estramustine phosphate which is derived from a secondary alcohol.\textsuperscript{[159,160]} Nevertheless, accumulation and following precipitation of poorly soluble parent drugs on their way to absorption area should be taken into consideration upon fast bioconversion of prodrug by ALP. Heimbach et al. have addressed such enzyme mediated-precipitation of phosphate prodrugs using TAT-59 (miproxifene-phosphate), fosphenytoin, and estramustine phosphate and they concluded that solubility of parent drug, initial prodrug concentration, required dose and extent of enzyme activity are crucial factors in terms of precipitation and should be considered carefully in the prodrug development process.\textsuperscript{[161]}

Polymers of phosphate monoesters have also been introduced to the market as prodrugs. Polyestradiolphosphate is such an example comprising of 13 repeating units of estradiol phosphate which is used for prostate cancer therapy.\textsuperscript{[162]} In the case of intramuscular injection of this macromolecular drug, it is stored in the spleen and liver afterward, enables a slow release of parent drug upon enzymatic cleavage by ALP exhibiting a depot action.\textsuperscript{[163]}

Apart from the phosphate monoesters derived from primary alcohols, thiophosphates are also subject to ALP-activated prodrug strategies. For instance, amifostine has been marketed under the name of Ethyol and it is used for relieving the cytotoxic effects caused by chemotherapy and radiotherapy in cancer patients.\textsuperscript{[164]} Amifostine is transformed to its parent drug WR-1065 by cleavage of $\text{P}―\text{S}$ bond upon contact with membrane-bound ALP and uptaken by healthy cells into 100-fold higher extent compared with cancer cells due to deficiency of ALP in tumor tissues. Following activation, WR-1065 binds free radicals generated by chemotherapeutics like cisplatin or radiotherapy generating a protection for healthy cells.\textsuperscript{[165]}

In addition to conventional prodrug strategies utilizing single prodrugs, ALP was the first enzyme exploited for antibody-directed enzyme prodrug approach (ADEPT) to enable a local action of the enzyme in combination with different prodrugs such as etoposide phosphate.\textsuperscript{[166]} In this set-up, a monoclonal antibody–ALP conjugate is administrated together with the prodrug and this combination binds to antigen-positive tumor cells enabling a site-specific action.\textsuperscript{[167]} However, these approaches with ALP remained limited with preclinical studies so far due to activation of prodrugs also in other parts of the body and resulting toxicity.\textsuperscript{[168]}

### 4. ALP-Responsive Phosphate Bearing Excipients

Encouraged by the clinical and commercial success of phosphate prodrugs being activated by ALP, the same strategy was transferred to various pharmaceutical excipients bearing phosphate groups or being phosphorylated in order to design smart delivery systems as summarized in Figure 6 and Table 4.

![Figure 6](https://www.advancedsciencenews.com)
Table 4. Phosphate bearing excipients and nanosystems stimulated by ALP (6-PGA; 6-phosphogluconic acid, ALP; alkaline phosphatase, ChS; chondroitin sulphate, CMC; carboxymethyl cellulose, CP; choline phosphate, CS; chitosan, D-Arg9; polyarginine, CSSAP; phosphorylated chitosan-stearic acid conjugates, DDS; drug delivery systems, DGPP; Dioctanoyl glycerol pyrophosphate, DOCP; 2-(3,3-Bis(oleoyloxy)propyl)dimethylammonio)ethyl hydrogen phosphate, DOPE; dioleoyl phosphatidyl ethanolamine, DSPE-PEG 2000, 1,2-Distearoyl-sn-glycero-3-phosphoethanolamine-N-(amino(poly-ethylen glycol))-2000), G6P; glucosamine 6-phosphate, HPS; hydroxypropyl starch, IPC; inverse-phosphocholine, mPEG-b-PpY; methoxy poly(ethylene glycol)-block-poly(phosphotyrosine) diblock copolymer, N-Ac-AA-DOPE; N-acetyl alanine alanine 1,2 Dioleoyl-sn-glycero-3-phosphatidylethanolamine, NPs; nanocomplexes, NPs; nanoparticles, p-12-AD; phosphorylated-12-Amino-1-dodecanol, p-Ser-OA; 2-ammonium-3-(octadec-9-en-1-ylamino)-3oxopropyl hydrogen phosphate salt PA; 1,2-dipalmitoyl-sn-glycero-3-phosphatidic acid sodium, PCL1; PEgylated cleavable lipopeptide1, pDNA; plasmid DNA, PEG-Suc; polyethylene glycol-mercaptopusuccinic acid, PEG-Pyr; Polyethylene glycol-phosphotyrosine, PEI; poly ethylenimine, Pho; phosphoserine, pHPMA; N-(2-hydroxypropyl) methacrylamide copolymer, PLGA; Poly(lactic-co-glycolic acid), POAP; N,N'-bis(polyoxymethylene)oleylamine, PolyP; sodium polyphosphate, PTP; phosphatase-triggerable lipid-based particles, PTyr; phosphorytysine, p-Tyr; phosphorylated tyramine PTyr-ODA; phosphorylated tyrosineoctadecylamide, R8; octa-arginine, R12; poly-l-arginine, SA-p-Tyr; stearic acid phosphotyrosine amide, SEDDS; self-emulsifying drug delivery systems, TPP; tripolyphosphate).

| ALP triggered factors | Chemical structure of ALP triggered factors | Nanosystem type | Before ALP stimuli | After ALP stimuli | Refs. |
|----------------------|---------------------------------------------|-----------------|--------------------|------------------|------|
| **Polyphosphates**   |                                             |                 |                    |                  |      |
| Polyphosphate (polyP) | ![Polyphosphate structure](image)           | PEI-PolyP NPs   | −9.14 mV           | −1.75 mV         | [185]|
| **Tripolyphosphate (TPP)** | ![Tripolyphosphate structure](image) | CS-TPP NPs     | nm (4)             | Nm               | [6]  |
| **Phytic Acid**      | ![Phytic Acid structure](image)            | Nanoemulsions   | −14.1 mV           | +4.2 mV          | [135]|
|                      |                                             | Nanoemulsions   | −9.9 mV            | −2.6 mV          | [135]|

(Continued)
Table 4. (Continued).

| ALP triggered factors | Chemical structure of ALP triggered factors | Nanosystem type | Before ALP stimuli | After ALP stimuli | Refs. |
|-----------------------|---------------------------------------------|-----------------|---------------------|-------------------|-------|
| Phosphorylated polymers |                                            |                 |                     |                   |       |
| Branched PEI 6-PGA   | ![Branched PEI 6-PGA Structure](image)     | PEI 6-PGA-CMC NPs | −6.44 mV           | +2.8 mV           | [95]  |
|                       | ![Branched PEI 6-PGA Structure](image)     | PEI 6-PGA-pDNA NCs | +3.25 mV           | +8.26 mV          | [204] |
|                       | ![Branched PEI 6-PGA Structure](image)     | PEI 6-PGA-pDNA NCs | +2.68 mV           | +6.96 mV          |       |
| Linear PEI 6-PGA     | ![Linear PEI 6-PGA Structure](image)       |                 |                     |                   |       |
|                       | ![Linear PEI 6-PGA Structure](image)       |                 |                     |                   |       |
| PEG-p-Tyr             | ![PEG-p-Tyr Structure](image)              |                 |                     |                   | [5]   |
| mPEG-b-PpY            | ![mPEG-b-PpY Structure](image)             | Nanocomplex     | −71.2 mV            | −28.7 mV          | [224] |

(Continued)
| ALP triggered factors | Chemical structure of ALP triggered factors | Nanosystem type | Before ALP stimuli | After ALP stimuli | Refs. |
|-----------------------|--------------------------------------------|-----------------|--------------------|------------------|------|
| CMC p-Tyr             | ![CMC p-Tyr Chemical Structure](image)      | CS P-Tyr-CMC p-Tyr NPs | −7.77 mV           | +8.10 mV         | [201] |
|                       |                                            | CS P-Tyr-CMC p-Tyr p-DNA NPs | −9 mV             | +9 mV            | [64]  |
| CS p-Tyr              | ![CS p-Tyr Chemical Structure](image)      | CS P-Tyr-CMC p-Tyr p-DNA NPs | −7.77 mV           | +8.10 mV         | [201] |
|                       |                                            | CS P-Tyr-CMC p-Tyr p-DNA NPs | −9 mV             | +9 mV            | [64]  |
| CMC G6P               | ![CMC G6P Chemical Structure](image)       | CMC 6GP-PEI NPs | −3 mV             | +4 mV            | [202] |
| CSSAP                 | ![CSSAP Chemical Structure](image)         | Micelles        | −20 mV            | −9 mV            | [64]  |
| Phosphorylated HPS    | ![Phosphorylated HPS Chemical Structure](image) | SEDDS           | −6.5 mV           | +1 mV            | [193] |
Table 4. (Continued).

| ALP triggered factors                      | Chemical structure of ALP triggered factors | Nanosystem type | Before ALP stimuli | After ALP stimuli | Refs. |
|-------------------------------------------|---------------------------------------------|-----------------|--------------------|-------------------|-------|
| ß-Cyclodextrin-6A-phosphate                | ![Chemical structure](image1.png)           | Liposomes (PTP) | −17.3 mV           | −7.5 mV           | [196] |
| Lipidated phosphorylated fusion peptide   | ![Chemical structure](image2.png)           | PLGA-PEG-R8-Pho NPs | −1.87 mV           | +7.37 mV          | [207] |
| DSPE-PEG2000-Pho                           | ![Chemical structure](image3.png)           | SEDDS           | −12 mV             | +5.3 mV           | [96]  |
| Phosphorylated surfactants                 | ![Chemical structure](image4.png)           | SEDDS           | −11.53 mV          | −2.04 mV          | [221] |
| p-Ser-OA                                  | ![Chemical structure](image5.png)           | SEDDS           | −9.37 mV           | +0.35 mV          | [222] |

(Continued)
Table 4. (Continued).

| ALP triggered factors | Chemical structure of ALP triggered factors | Nanosystem type | Before ALP stimuli | After ALP stimuli | Refs. |
|-----------------------|--------------------------------------------|-----------------|--------------------|-------------------|-------|
| p-Tyr | ![Chemical structure of p-Tyr](image1) | Nanoemulsions | −8.40 mV | +1.2 mV | [223] |
| SA-p-Tyr | ![Chemical structure of SA-p-Tyr](image2) | SEDDS | −14 mV | +2 mV | [225] |
| POAP | ![Chemical structure of POAP](image3) | SEDDS | −15.1 mV | +6.5 mV | [218] |
| PNPP | ![Chemical structure of PNPP](image4) | SEDDS | −33.7 mV | +8.2 mV | [104] |
| PME | ![Chemical structure of PME](image5) | SEDDS | −13 mV | +9 mV | [81] |
| Phospholipids | ![Chemical structure of Phospholipids](image6) | SEDDS | −7 mV | +9 mV | [81] |
| Phosphatidic acid | ![Chemical structure of Phosphatidic acid](image7) | SEDDS | −1.28 mV | +0.52 mV | [67] |
| Phospholipids | ![Chemical structure of Phospholipids](image8) | Nanoemulsion | −60 mV | −20 mV | [81] |

(Continued)
Table 4. (Continued).

| ALP triggered factors       | Chemical structure of ALP triggered factors | Nanosystem type | Before ALP stimuli | After ALP stimuli | Refs. |
|-----------------------------|---------------------------------------------|-----------------|--------------------|-------------------|-------|
| Cholesterol phosphate       | ![Chemical structure of Cholesterol phosphate](image1)'s chemical structure | Liposomes       | nm                 | nm                | [215] |
| DOCP                        | ![Chemical structure of DOCP](image2)'s chemical structure | Liposomes       | nm                 | nm                | [214] |
| DGPP                        | ![Chemical structure of DGPP](image3)'s chemical structure | SEDDS           | −32 mV             | −31 mV            | [81]  |
| Surface phosphorylated NCs  | ![Chemical structure of Surface phosphorylated NCs](image4)'s chemical structure | SEDDS           | −24 mV             | −18 mV            | [81]  |
| Enzymatic surface phosphorylation of NCs | ![Chemical structure of Enzymatic surface phosphorylation of NCs](image5)'s chemical structure | CS-ChS NPs      | −12.4 mV           | −1.2 mV           | [200] |
| ALP triggered factors                        | Structure of ALP triggered factors | Nanosystem type       | Before ALP stimuli                                      | After ALP stimuli                                      | Refs. |
|---------------------------------------------|-----------------------------------|-----------------------|---------------------------------------------------------|-------------------------------------------------------|-------|
| **Self-assembling nanofibers and hydrogels**|                                   |                       |                                                        |                                                        |       |
| Fmoc-pY                                    | Solution                          | Hydrogels contain nanofibrils in widths of 20–25 nm             | [208]                                                   |                                                       |       |
| Nap-pβ1-HPHg-β1-HPHg-pY                    | Solution                          | Hydrogel              | [209]                                                   |                                                       |       |
| Nucleobase-FF                               | Solution                          | Hydrogels contain nanofibers in widths of 9–20 nm              | [210]                                                   |                                                       |       |
| Nap-FFpY                                   | Solution                          | Hydrogel/nanonet      | [211]                                                   |                                                       |       |
| Nap-pFpY-βγpY, Nap-pFpY-γpY-βγpY            | Solution                          | Hydrogels contain nanofibers in widths of 5–7 nm                | [212]                                                   |                                                       |       |
| Nap-pFpY-γpY-OMe, Nap-pFpY-NHMe             | Solution contains                 | Hydrogels contain nanofibers with diameters of 6–14 nm          | [213]                                                   |                                                       |       |
| Nap-FFpY                                   | Solution                          | Hydrogels contain nanofibers in a width of 29 nm                | [214]                                                   |                                                       |       |
| Taxol-Nap-FFpY                             | Solution                          | Hydrogels contain nanofibers in widths of 15–25 nm             | [215]                                                   |                                                       |       |
| Taxol-FFFpY                                | Solution                          | Hydrogels contain nanofibers in widths of 2.5–4 nm             | [216]                                                   |                                                       |       |
| Taxol-Nap-FFFpY or Taxol-Nap-pFpY-γpY       | Solution                          | Hydrogels contain nanofibers in widths of 8–9 nm                | [217]                                                   |                                                       |       |
| Etoposide phosphate-Nap-FFFpY               | Solution                          | Hydrogels of nanofibers                                        | [218]                                                   |                                                       |       |
| Fluorophore ICG-Nap-FFFpY                  | Micelles solution                 | Hydrogels contain nanofibers in widths of 204–210 nm           | [219]                                                   |                                                       |       |
| Fluorophore NBD-Nap-pFpY                   | Solution                          | Hydrogels contain nanofibers with diameters of 7–9 nm          | [220]                                                   |                                                       |       |
| Fluorophore NBD-pFpY                       | Solution                          | Hydrogels contain nanofibers in widths of 50 nm                 | [221]                                                   |                                                       |       |
| NSAID-pFpY                                 | Solution                          | Hydrogels contain nanofibers in widths of 3.8–4.3 nm           | [222]                                                   |                                                       |       |
| TPEpy-pY                                   | Solution                          | LCST-type hydrogels                                            | [223]                                                   |                                                       |       |
| Chemiluminescence-Fmoc-FFFpY               | Solution                          | Hydrogels of nanofibers                                        | [224]                                                   |                                                       |       |
| poly(DEGMA-co-Phos-HEMA)                   | Solution of spherical aggregates  | Hydrogels of nanofibers                                        | [225]                                                   |                                                       |       |
| N-(fluorenylmethoxycarbonyl)-glucosamine-6-phosphate |                       | Hydrogels contain nanofibers with an average diameter of 10 nm | [226]                                                   |                                                       |       |
| Reversiblesol-gel                          |                                   |                       |                                                        |                                                        |       |
| Nap-FFGePy                                  | Solution                          | Sol (1)-gel (2) transition by ALP formed hydrogels of nanofibers with the diameters of 28 ± 5 nm; Gel (2)-sol (1) transition by kinase; Sol (1)-gel (3) transition by ALP formed hydrogels of nanotubes with diameters of 18 ± 3.5 nm | [227]                                                   |                                                       |       |
| KRRASVAGK-NH2                               | Spherical micelle-like aggregates solution | Sol (1)-gel (2) transition by ALP formed hydrogels of cylindrical β-sheet nanofibers with a minimum at 218 nm; Gel (2)-sol (1) transition by kinase formed solution of random coil with a minimum at 196 nm | [228]                                                   |                                                       |       |
| Fmoc-K(FITC)FFpY                            | Fluorescence solution             | Hydrogels contain fluorescence “turn-off” nanofibers with an average diameter of 10 nm | [229]                                                   |                                                       |       |

(Continued)
Table 4. (Continued).

| ALP triggered factors | Structure of ALP triggered factors                                                                 | Nanosystem type                          | Before ALP stimuli                                                                 | After ALP stimuli                                                                 | Refs.       |
|-----------------------|---------------------------------------------------------------------------------------------------|------------------------------------------|------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------|-------------|
| **Multiresponsive hydrogels** |                                                                                                  |                                           |                                                                                     |                                                                                    |             |
|                   | Fmoc-pY-P(Pro)x-OH combine the ALP- and thermal-responsive properties                                | Solution                                 | ALP-induced self-assembly micelles collapse above LCST to form small mesoglobules with a diameter of 21 nm |                                                                                    | [211]       |
|                   | Fmoc-pY-OMe contained both an ALP- and carboxylesterases-cleavage site (i.e., carboxyl methyl ester) | Solution                                 | Hydrogels contain nanofibrils of 8 ± 2 nm in diameter when incubation with ALP; Solution contains small particles of 7 ± 2 nm in diameter when co-incubation with ALP and esterase |                                                                                    | [241]       |
| **Aggregating and disaggregating systems** |                                                                                                  |                                           |                                                                                     |                                                                                    |             |
| **Aggregating systems** |                                                                                                  |                                           |                                                                                     |                                                                                    |             |
|                   | Magnetic nanoparticles Fe₃O₄-pY                                                                | Magnetic nanoparticles                  | Solution                                                                           | Aggregates of magnetic nanoparticles on cell surfaces                            | [242]       |
|                   | Nanoparticles decorated pY or pY                                                               | Nanoparticles                            | Solution contains small aggregates with an average size of 500 nm                  | Aggregates of nanoparticles on cell surfaces                                     | [243]       |
|                   | Fluorophore NBD-FYK-pY conjugated triphenylphosphonium                                        | Nanoparticles                            | Solution of tiny nanoparticles                                                     | Aggregated hollow colloids                                                       | [244]       |
|                   | Gold nanoparticles decorated CREKA-YPFK(Nph)                                                  | Gold nanoparticles                      | Solution contains spherical nanoparticles with an average size of 30 nm              | Aggregates of gold nanoparticles with a wide size distribution from 15 to 172 nm | [245]       |
|                   | Gold nanoparticles pY-AuNPs-doxorubicin                                                        | Gold nanoparticles                      | Solution contains nanoparticles with a core size of 11 nm                           | Aggregates of gold nanoparticles                                                  | [246]       |
|                   | Ternary cyclodextrin-liposome constructed from silibinin-β-cyclodextrin complex, phosphatidyl choline, and MPEG-2000-DSPE |                     | Solution                                                                           | Aggregates of nanoassemblies with a diameter of 174 nm                           | [247]       |
|                   | Solid lipid nanoparticles decorated phosphate ester surfactant and octadecylamine              | Solid lipid nanoparticles                | Solution contains nanoparticles with a mean size of 120 nm                          | Solid lipid nanoparticles aggregates in the size of 5–8 μm                         | [7]         |
| **Disaggregating systems** |                                                                                                  |                                           |                                                                                     |                                                                                    |             |
|                   | Block copolymers PEG-b-PLKC conjugated ATP                                                      |                                           | Solution of self-assembled aggregates with an average diameter of 70–80 nm         | Disassembly of the spherical aggregates                                           | [248]       |
|                   | Calixarene derivative AC4AH conjugated ATP                                                     |                                           | Solution of spherical particles with a diameter of 200–300 nm                      | Disassembly of the spherical particles                                             | [249]       |

**nm**: not mentioned.
4.1. Polyphosphates

Polyphosphates have been utilized in biomedicine for various purposes such as for crosslinking of nanoparticles,[178] wound dressing,[179] tissue engineering,[180] and drug delivery.[181] The polyphosphate term covers a broad range of compounds that are obtained from acidic monophosphate units by mainly condensation reactions and can be classified into the following subcategories: linear polyphosphates, cyclic metaphosphates, and cross-linked, or branched polyphosphates.[182] They have a general formula as $\text{Me}_{(n+2)}\text{P}_n\text{O}_{(3n+1)}$ where Me represents metal cation. The degree of polymerization ($n$) can vary from 2 to 106 generating a range of products from simple pyro-, triply-, and tetrapolypophosphates to high molecular weight branched polyphosphates.[183] As a subcategory sodium polyphosphates being used as pharmaceutical excipients such as sodium tripolyphosphate (TPP) is accepted as generally recognized as safe (GRAS) by the FDA.[184] These structures can be obtained by condensation of two or more sodium phosphate units to form a chain or cyclic structures under high temperature by elimination of water. For the production of sodium tripolyphosphate ($n = 3$), two moles of $\text{Na}_2\text{HPO}_4$ and one mole of $\text{NaH}_2\text{PO}_4$ are condensed under 300–550 °C. By the increase of $\text{NaH}_2\text{PO}_4$ ratio and temperature up to 700–800 °C, glassy polyphosphates can be obtained upon fast cooling of melted structure with higher degrees of polymerization, such as Graham’s salt ($n = 25$).[182,183] The obtained polyphosphates have a polyanionic nature enabling high cation-binding capacity and can be utilized for the preparation of nanoparticles containing cationic polymers such as chitosan or polyethylene imine (PEI) via coacervation[6–185] and as coating material.[105]

Polyphosphoesters being polyphosphates with higher molecular weight and side-chain modifications can be obtained by functional group conjugations to the central polyphosphate backbone due to pentavalent phosphorus atom enabling the attachment of various functional groups. These polymers can be synthesized by mainly polycondensation and also by polyaddition, transesterification, and ring-opening polymerization methods[186] and are promising excipients since they are easy to modify and comparatively stable. Furthermore, they are biocompatible and biodegradable in the bioenvironment due to enzymatic cleavage going hand in hand with low cytotoxicity.[187] ALP-responsive polyphosphoesters were obtained by coupling the polyphosphate to various functional groups including polyethylene glycol[188,189] or sulfobetaine.[190]

Biopolyphosphates were also exploited as ALP-responsive excipients. In contrast to polyphosphates that are synthesized at high temperatures, biopolyphosphates are formed by enzymatic reactions under mild conditions of living organisms such as plants, bacteria, fungi, and animals.[191] Phytic acid, for instance, is a biopolyphosphate that can be obtained from plants and used as a coating material for ALP-activated drug delivery systems.[105]

4.2. Phosphorylated Hydrophilic Polymers

Various types of hydrophilic polymers have been phosphorylated by different techniques to be used in biomedical systems working upon ALP stimuli. As illustrated in Figure 7, there are three ways to covalently attach phosphate groups on polymers; direct phosphorylation of polymers using highly reactive phosphoryla-
tion reagents (i), enzyme-mediated phosphorylation (ii), and attachment of already phosphorylated ligands to polymers (iii).

In order to attach phosphate groups directly to polymer backbones and to ultimately generate ALP-responsive polymers, in particular polymeric excipients bearing hydroxyl groups are of interest being transformed to phosphate monoesters with phosphoryl donors such as orthophosphoric acid, phosphorus pentoxide, or phosphor chloride. Datta et al., for instance, synthesized phosphorylated polyvinyl alcohol upon esterification reaction of the hydroxyl groups with orthophosphoric acid.[192] ALP-responsive phosphorylated polysaccharides were synthesized with phosphorus pentoxide, the highly reactive anhydride form of phosphoric acid. Griesser et al. phosphorylated starch and hydroxypropyl starch (HPS) using phosphorus pentoxide, providing 119.4 ± 5.2 and 259.3 ± 1.2 μmol of phosphate attachment per g of phosphorylated starch and HPS, respectively.[193] The combination of phosphorus pentoxide and methanesulfonic acid has been utilized to phosphorylate chitin and chitosan derivatives in high yields. Methanesulfonic acid acts as a solvent for chitin and chitosan and more importantly covers primary amino groups of the polymers via ionic interactions driving the phosphorylation reaction into the hydroxyl functionality.[194, 195] In order to generate a phosphorylated amphiphilic polymer, for instance, phosphorylated stearic acid–chitosan conjugates were obtained by using phosphorus pentoxide in methanesulfonic acid, resulting 1066 ± 56 μmol of phosphate coupling per g of stearic acid-chitosan compound.[64] More rarely, phosphor chloride was utilized as phosphorylating reagent to obtain ALP-responsive phosphorylated hydrophilic polymers. β-Cyclodextrin and polypeptides containing arginine units were phosphorylated by phosphoryl chloride and investigated as ALP substrates.[196, 197]

Kinases play an important role in phosphorylation of biomolecules driving various cellular processes. Enzymatic phosphorylation by kinases in the presence of high-energy donors such as adenosine phosphates has been discovered as an alternative pathway to generate phosphorylated polymers. Yang et al., for instance, phosphorylated a pentapeptide (naphthalene (Nap)–FFGEY) by tyrosine kinase in the presence of adenosine triphosphate (ATP) and dephosphorylation was achieved by ALP enabling a kinase/phosphatase switch.[198] In another study, Winkler et al. phosphorylated a 25 kDa silk protein via phosphoryl transfer by cyclic adenosine monophosphate (AMP)-dependent kinase to the serine units and observed dephosphorylation upon incubation with calf IAP.[199] Apart from peptides and proteins, Nazir et al. achieved the enzymatic phosphorylation of chitosan and chondroitin sulfate by hexokinase using ATP as the energy source. In this study, nanoparticles were formed between the cationic and anionic polysaccharides via coacervation followed by surface phosphorylation of these particles utilizing the enzyme.[200]

Following the approach of the attachment of already phosphorylated ligands to polymers, phosphorylated amino acids and phosphorylated amino acid like-ligands are widely used. Carbodiimide chemistry is a common method to form amide bonds between carboxyl groups of the polymer and primary amine groups of such phospho-amino-compounds or vice versa, through an initial activation of carboxyl moieties by EDC (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide) (Figure 8). Upon addition of EDC, an amine-reactive intermediate product, namely, O-acetylsoirea forms and by a nucleophilic attack from primary amine groups to the activated carboxyl groups, amide bond forms in acidic conditions. Accordingly, phosphorylated chitosan and carboxymethyl cellulose (CMC) were obtained by covalent attachment of phosphotyrosine at pH 5.[201] In another study, glucoseamine 6-phosphate (G6P) was used as a phosphorylating agent to phosphorylate CMC in the presence of EDC at pH 5.[202] However, O-acetylsoirea intermediate is an unstable intermediate and in case of insufficient interaction with amine moieties, it can be degraded by hydrolysis lowering the yield for amide bond formation. In order to increase the amide bond formation and thus phosphorylation efficiency, usage of N-hydroxysuccinimide (NHS) together with EDC is another common approach. Within this approach, EDC introduces the NHS to carboxyl moieties and more stable NHS esters form as amine-reactive stable intermediates forming the final amide bonds with primary amines at physiological pH resulting in high yields.[203]

Phosphorylated branched polyethylene imine for instance was synthesized by covalent coupling of 6-phosphogluconic acid (6-PGA) in the presence of EDC and NHS.[203] In a follow-up study, a higher phosphorylation efficiency was shown for branched PEI-6-PGA compared with linear PEI-6-PGA due to relatively higher primary amine content accessible for attachment of 6-PGA.[204] Similarly, Bonengel et al. synthesized phosphorylated polyethylene glycol (PEG) by forming an amide bond between amine groups of PEG–diamine and carboxylic acid moieties of phosphotyrosine using EDC and NHS.[205] By another research group, phosphorylated naphthalene capped polypeptides were synthesized using NHS together with N,N′-dicyclohexylcarbodiimide (DCC) that is an organic solvent-soluble analog of EDC to activate carboxylic acid groups of terminal amino acids of the polypeptides. Following the conjugation of phosphotyrosine into the activated terminal carboxyl moieties of naphthalene capped polypeptides, ALP-activated hydrogelators were formed.[205, 206] Alternatively, commercially available NHS esters of polymers can be utilized in order to accelerate the phosphorylation process. Wu et al., phosphorylated a lipid-containing PEG upon covalent attachment of O-phospho-L-serine into commercially available 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(poly(ethylene glycol))-2000] (DSPE-PEG2000)-NHS.[207]

Solid-phase peptide synthesis method was also utilized to design ALP-responsive functional polypeptides containing fluororemethoxycarbonyl-phosphotyrosine (Fmoc-pY) that has a self-assembly property upon ALP stimuli.[208] Fmoc solid-phase peptide synthesis is a method that enables peptide chain formation and further purification on a solid support such as a resin by stepwise addition of amine group protected Fmoc-amino acids. During the synthesis process that occurs in resin, amino acids are deprotected and carboxyl groups are activated for amide bond formation.[209] Kiran et al. for instance generated a phosphorylated polypeptide namely, Nap-Phe-Phe-Tyr(H₂PO₄)-OH by Fmoc solid-phase peptide synthesis using 2-chlorotrityl chloride resin.[210] In another study, authors designed a hybrid thermolabile ALP-responsive polymer by a coupling reaction of Fmoc-pY to 11-azido-3,6,9-trioxadecan-1-amine to form Fmoc-pY-azide by solid-phase peptide synthesis method.[211]
Figure 8. Phosphorylation of hydrophilic polymers by carbodiimide chemistry. A) Synthesis of phosphorylated hydrophilic polymers by a commonly used phosphorylated amino acid, phosphotyrosine. Modified with permission. [66] Copyright 2017, Future Medicine LTD. B) Activation of carboxyl groups by 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) or N,N'-dicyclohexylcarbodiimide (DCC) (1) to form amide bonds upon addition of primary amines (2,4). In order to obtain more stable amine reactive intermediates and increase the efficiency of amide bond formation, N-hydroxysuccinimide (NHS) can be utilized (3). R₁-COOH and R₂-NH₂ represent the carboxyl and the primary amine sources respectively, being as polymers or phospho-amino compounds.
4.3. Phosphorylated Lipids

Up to date, a variety of phosphorylated lipids has been utilized for drug delivery and diagnostics including natural and synthetic phospholipids and phosphorylated lipids.

Phospholipids are a class of lipids that contain a phosphate group, glycerol and fatty acids. They are in particular of relevance for the design of lipid-based NCS systems due to their amphiphilic nature leading to their accumulation on oil–water interfaces with the polar phosphate substructure being oriented to the aqueous phase. Furthermore, they are biodegradable and generally recognized as safe (GRAS). Phosphatidic acid (1,2-dipalmitoyl-sn-glycerol-3-phosphatic acid, PA) has been exploited as ALP-responsive excipient for the design of charge reversal lipid-based NCS. [212-213]

Phospholipids exhibiting a choline substructure cannot be properly cleaved by ALP, as the phosphate substructure is a nonterminal diester. In order to address this issue, inverse-phosphocholine lipids were designed by Perttu et al. [214] These inverse phospholipids exhibiting a terminal phosphate group were synthesized using trialkylphosphites as phosphorylating reagents. The authors observed the susceptibility of these inverted phosphocholines for ALP and cleavage of phosphate groups generated cationic surfactants by the remaining choline structure.

In another study, a phospholipid containing pyrophosphate structure namely, dioctanoyl glycerol pyrophosphate (DGPP) was investigated as a substrate for ALP. Phosphate cleavage from this excipient upon incubation with ALP was compared with PA and 2-(2,3-bis(oleoyloxy)propyl)dimeethylammonio)ethyl hydrogen phosphate (DOCP). Accordingly, phospholipids having long lipid chains, i.e., PA and DOCP displayed ~10-fold less phosphate cleavage by ALP compared with DGPP having shorter lipid chains likely due to inhibitory effects of long lipid chains on ALP activity. [81] Similar inhibitory effects of long-chain phospholipids such as dimyristoyl lecithin (C14) and palmitoyl lysolecithin (C16) were also observed on ALP activity unlike a short-chain phospholipid, dicaproyl lecithin (C6) suggesting that the lipid part was responsible for the inhibitory effect. Furthermore, they observed that this effect was not dependent on the phosphate ester region since there was still an inhibition in the case of a non-phospholipid structure, myristic acid (C14). [86]

Apart from phospholipids, phosphate monoesters of cholesterol were synthesized upon phosphorylation of diols placed on the 3-β position of the cholesterol ring by tetranbutylammonium phosphate which is a reagent used for the synthesis of phosphate monoesters from alcohols with high yields. Accordingly, within 24 h 40% of the cholesterol phosphate was hydrolyzed by ALP. [215]

More recently, Jin et al. designed a novel hybrid oligonucleotide probe (DNA-lipid-P) consisting of DNA and a phosphorylated lipid aiming to achieve an ALP-dependent cell membrane attachment of the nucleic acid. The probe consists of DNA, N,N-didodecylamine as hydrophobic linker and terminal phosphates linked to hydrophilic lipid chains as ALP substrate. DNA-lipid-P synthesized using solid-phase oligonucleotide synthesis with phosphoramidite method [216] that enables generation of oligonucleotides by chemical routes from nucleotides and further purification on solid supports such as controlled pore glass (CPG). [217]

In order to achieve the phosphorylation, a modified phosphoramidite namely, 4,4′-dimethoxytrityl-(DMT)-protected hydroxyl was synthesized that enables terminal phosphate functionalization of lipid chains. [216]

4.3.1. PEGylated Phosphorylated Lipids

Steric hindrances can affect phosphate cleavage from phosphorylated lipids by ALP, especially when they are located on the surface of certain DDS such as nanoemulsions together with other excipients such as PEGylated surfactants. The alignment of PEGylated surfactants on the oil–water interfaces of lipid-based NCS results in the formation of a PEG-corona that seems to hinder ALP to cleave underlying phosphate substraces. Therefore, to design functional phosphorylated lipids for ALP-responsive PEG containing lipid-based DDS, hydrophilic spacers can be utilized to extend the hydrophilic part of phosphorylated lipids to locate the phosphate group on the outer surface. Wolf et al. synthesized a PEGylated phosphorylated surfactant having two PEG chains as hydrophilic linkers between the terminal phosphate group and hydrophobic part namely N,N′bis(polyoxyethylene)oleylamine bisphosphate (POAP) using pyrophosphoric acid. [218] Accordingly, within 4 h 50% of the attached phosphates were released from POAP upon incubation with bovine intestinal ALP. In order to eliminate the toxic effects derived from tertiary amine structure of POAP, Kurpier et al. used polyoxyethylene (9) nonylphenol monophosphate ester (PNPP) as phosphorylated surfactant and upon incubation with bovine intestinal ALP within 4 h 92% of total phosphate was cleaved off which is almost twofold higher than POAP. [104] Apart from the formulation-related factors for this enhancement, structure differences of the surfactants are playing an important role. Due to double-chain structure of POAP, a steric hindrance might take place leading to an increased phosphate release in the case of PNPP having a single PEG chain (Table 4). Furthermore, the long lipid tail of POAP (C18) might have an inhibitory effect on the activity of ALP as similar effects were observed for phospholipids. The structure-cleavage kinetics of PEGylated phosphorylated surfactants were also evaluated in a comparative study investigating the phosphate cleavage from five different phosphorylated surfactants upon ALP treatment. Within this study, a higher extent of phosphate cleavage was observed in case of PEGylated phosphorylated surfactants compared with phospholipids having no ethoxylate linkers. This effect was derived from inductive effects of remaining PEG groups of the surfactants upon cleavage of phosphate groups enhancing the ALP activity and hence phosphate release. [81] The activatory effect of PEGs on bovine IAP was previously indicated. [114] Correspondingly, activatory effect of various PEGs with different chain lengths was investigated and an increase in the hydrolysis of an ALP substrate, pNPP was observed with increasing chain length of free PEGs up to PEG2000. Although the sites of ALP that PEG binds could not be identified, a higher enzymatic activity was also observed in the case of PEGylated ALP. [114] Indeed, activatory effects of PEG groups were reported for other enzymes as well, and PEGylation was utilized as a method to increase stability as well as activity of enzymes such as lipase and cellulase depending on the molecular weight of chosen PEG and ionic composition of the media. [219]
4.3.2. Flip-Flop Lipids

Flip-flop lipids have the ability to convert their orientation on the oil–water interface by change of their structure through changes in pH, temperature, ion content, or via enzymatic cleavage. This phenomenon has been widely studied for movement of phospholipids in cell membrane bilayers and utilized in the biomedical field. For instance, phosphatidylserine is used as a well-known biomarker for apoptosis due to its movement from inner core to the outer surface of the cell membrane by scramblase and inhibition of flippase during apoptosis. Once cell surfaces are covered with phosphatidylserine, they can be recognized by macrophages and engulfed readily. In the case of ALP-activated flip-flop surfactants, the change in orientation is caused by cleavage of the phosphate moiety and the flip out of the remaining polar group which is an amino structure in most cases (Figure 9). Salimi et al. were likely the first synthesizing an ALP-activated flip-flop surfactant by amide bond formation between tyrosine and octadecylamine followed by phosphorylation of hydroxyl groups with orthophosphoric acid (PTyr-ODA). Similarly, a flip-flop surfactant was synthesized via phosphorylation of serine-oleylamine by combination of phosphorus pentoxide and orthophosphoric acid (p-Ser-OA). These above-mentioned surfactants contain long lipid chains with a polar head containing both phosphate and amino substructures. Alternatively, Sharifi et al. synthesized comparatively small surfactants containing on the one head a phosphate group and on the other head a primary amino group by phosphorylation of 12-amino-dodecanol with phosphorus pentoxide (12-AD) and by phosphorylation of tyramine using phosphorus chloride.

5. ALP Related Drug Delivery Systems

By utilizing natural and synthetic phosphorylated excipients discussed in Section 5, various types of phosphate-functionalized DDS as illustrated in Figure 10 and Table 4 can be designed.

5.1. Charge Converting Delivery Systems

Surface charge plays a major role in the interaction of DDS with the biological environment. Due to the abundant polyanionic character of body fluids, membranes, and tissues, cationic DDS is hindered by electrostatic interactions to reach their target. At the target site, however, a cationic charge is desired to enhance cellular uptake of the carrier through electrostatic interactions with the anionic cell membrane. In order to address this so-called “polycation dilemma,” charge reversal DDS are utilized converting their charge from negative to positive at the target site. A promising approach to guarantee charge conversion at the target site is based on ALP. As a membrane-bound enzyme, it enables charge conversions from negative to positive values directly on the cellular membrane of target cells by the cleavage and release of anionic phosphate from the surface of different drug carrier systems. In this regard, charge reversion concept was applied to various polymeric or lipid-based DDS as illustrated in Figure 11.

Bonengel et al., for instance, employed the concept of charge conversion for the design of PEI NCs. ALP-responsive charge converting polymeric nanoparticles were formed by coacervation of negatively charged phosphorylated PEI and polycations. Although not all phosphate moieties were located on the surface of these systems, the total Δ9.24 mV change in zeta potential was observed. The limited accessibility of phosphate groups was in the following addressed by an enzymatic surface phosphorylation of chitosan–chondroitin sulfate nanoparticles. Similarly, charge converting polymeric nanoparticles were obtained by coacervation of various phosphorylated polymers with an oppositely charged polion by means of electrostatic complex formation. Alternatively, sodium polyphosphate (n = 25) was utilized to form polyelectrolyte complex nanoparticles with PEI as an ALP-responsive charge converting system. Furthermore, charge converting polymeric mi-
Figure 10. Alkaline phosphatase triggered drug delivery systems.

Figure 11. Examples of charge-reversal NCs activated by ALP.
Figure 12. SEDDS formulations containing different phosphorylated surfactants and their efficacy for phosphate cleavage by overcoming the PEG corona via ALP. Accordingly, highest extent of charge reversion was observed in case of SEDDS containing PNPP having the longest PEG spacer arm. Adapted with permission. [81] Copyright 2021, Elsevier.

celles were designed by using phosphorylated chitosan-stearic acid conjugates (CSSAP). [64]

A proof of concept for ALP-triggered charge converting delivery systems was also provided for lipid-based formulations and in particular self-emulsifying drug delivery systems (SEDDS) that are advantageous from the industrial point of view. The very first zeta potential changing SEDDS was developed by Suchaoin et al. with a charge conversion from just $-1$ mV to $+1$ mV. [67] Within this formulation, cationic surfactants were utilized to provide a positive charge upon cleavage of phosphate groups from phosphatidic acid by ALP. In the following studies, flip-flop surfactants having phosphate and amine groups on the same polar head group of the surfactants were incorporated into SEDDS and nanoemulsions. Within these systems, undesirable charge interactions such as ion pairing between cationic and anionic surfactants could be avoided using all-in-one flip-flop surfactants. This concept was applied by Salimi et al. using a phospholipid-like flip-flop surfactant and upon IAP treatment a charge conversion of SEDDS was achieved displaying a total $\Delta 17.5$ mV change in zeta potential. [96] In a following study, charge converting SEDDS containing another phospholipid-like flip-flop surfactant, namely, phosphorylated serine-oleylamine (p-Ser-OA) was developed. [221] The charge conversion upon ALP treatment was also reported for SEDDS containing a Janus-headed flip-flop surfactant (phosphorylated 12-amino-dodecanol, 12-AD) [222] and zwitterionic flip-flop surfactant loaded nanoemulsions. [223]

The composition of SEDDS and nanoemulsions is also crucial in terms of the proper positioning of phosphate groups on the surface for an effective enzymatic cleavage. Most SEDDS contain PEG-based emulsifiers such as poloxamers or polyethoxylated castor oil. [218] PEGylated emulsifiers form a PEG corona on the surface of SEDDS or nanoemulsions hindering the enzyme on phosphate cleavage. This corona effect was shown by Zaichik et al., as in the absence of PEGylated emulsifiers zeta potential changes of $\Delta 40$ mV upon contact with ALP were measured whereas nanoemulsions containing polyethoxylated-35 castor oil remained constant without any significant change in zeta potential due to the hindrance of PA. [213] Furthermore, a significant amount of phosphate was released from DOCP liposomes upon incubation with ALP due to absence of such a PEG corona on the surface. [214] In order to overcome PEG–corona, phosphorylated surfactants with PEG spacer arms were utilized and $\Delta 21.6$ mV and $\Delta 41.9$ mV changes in zeta potential were achieved for POAP [218] and PNPP [104] containing formulations, respectively. Further evidence was provided by comparing the phosphate release efficiency of phosphorylated surfactants having been incorporated into SEDDS as illustrated in Figure 12 upon ALP treatment. [85] A comparatively lower extent of phosphate cleavage was observed for SEDDS containing the surfactants having shorter hydrophilic linkers (DOCP and PA) due to insufficient length of hydrophilic linkers carrying terminal phosphate to the outer surface of PEG corona for an efficient cleavage by ALP leading to a charge conversion.

Mucosal tissues that have already been targeted with charge converting DDS are the intestinal, pulmonary, and ocular mucosa. As the mucus gel layer exhibits a net negative charge due to sialic and sulfonic acid moieties of mucins, negatively charged NCs can permeate it more rapidly, whereas, positively charged NCs are efficiently taken up by the epithelium due to heparin sulfate structures on its surface. [200] Therefore, charge converting NCs were utilized for mucosal drug delivery and in particular were broadly investigated for intestinal drug delivery. As such
NCs provide high mucus permeating properties and an improved cellular uptake, they are in particular of interest for oral delivery of BCS class 3 and 4 drugs, even as challenging drugs as therapeutic peptides and DNA/RNA based drugs might be successfully delivered by such systems. Wu et al., for instance, developed a charge converting oral delivery system for insulin by surface coating of poly(lactic-co-glycolic acid) with anionic phosphoserine and cationic octa-arginine. These NCs exhibited 2.37-fold higher permeation across the porcine intestinal mucus compared to poly(lactic-co-glycolic acid) octa-arginine NCs due to their negative surface charge. The uptake of these NCs by Caco-2 cells was almost 1.5-fold increased after phosphate cleavage using ALP. Furthermore, insulin-loaded charge converting NCs showed 1.9-fold higher oral bioavailability on diabetic rats. Additionally, Sharifi et al. designed charge reversible nanoemulsions containing phosphorylated tyramine (p-Tyra) on the surface of the oily droplets exhibiting even further improved mucus permeating properties. Within this study, p-Tyra loaded nanoemulsions permeated the porcine intestinal mucus into a 8.59-fold higher extent compared with dephosphorylated p-Tyra loaded nanoemulsions that were treated with ALP prior to the experiment. Moreover, a 2.4-fold higher uptake of p-Tyra loaded nanoemulsions by Caco-2 cells was indicated compared to the cells having an inhibited ALP activity proving that the cellular uptake of the developed nanoemulsions was ALP selective. Similar results were also reported for various charge converting NCs that aim an enhanced permeation through the intestinal mucus gel layer and an ALP-selective cellular uptake following the enzymatic cleavage of phosphate groups. Furthermore, charge reversal concept was utilized to achieve a successful gene delivery across mucous and epithelial barriers. In this regard, Bonengel et al. developed charge converting PCNA3-EGFP (green fluorescent plasmid) loaded PEI nanoparticles and they observed an ALP-selective transfection of Caco-2 as well as HEK-293 cells upon incubation of cells with the developed NCs. In another study, Leichner et al. developed ALP-responsive chitosan-tripolyphosphate nanoparticles to achieve the intestinal delivery of β-galactosidase that is required to metabolize the lactose for the individuals having lactase deficiency. Accordingly, the developed NCs provided a time-dependent release of β-galactosidase by overcoming the mucus barrier of porcine small intestine and following phosphate cleavage by mucus-underlying ALP. Furthermore, developed NCs were able to protect the β-galactosidase against trypsin degradation indicating an increased stability of the macromolecule toward undesired premature degradation by intestinal proteases.

Muco-inert NCs can permeate the mucus gel layer and reach the underlying epithelium. However, these NCs can also back diffuse into the mucus gel layer. In order to avoid the back diffusion of NCs, Bonengel et al. designed a novel surface coating material for charge converting NCs, a phosphorylated PEG to overcome the back diffusion effect in the mucus gel layer by conversion of a mucus-penetrating polymer to the mucoadhesive upon ALP-stimuli. Accordingly, phosphorylated PEG permeated the mucus gel layer three times faster than dephosphorylated polymer. Furthermore, interactions of the polymer with native mucus were investigated via rheological analysis. An enhanced dynamic viscosity was observed for the dephosphorylated polymer as well as for the unmodified PEG indicating that upon ALP treatment the polymer displays a mucoadhesive nature. The PEG derivatives are well-known and widely used due to their mucus-permeating properties. In order to overcome also the back-diffusion problem in mucosal environments the bifunctional phosphorylated PEG displaying switchable mucus permeating-mucoadhesive properties by ALP can be utilized for future charge converting DDS.

In case of pulmonary delivery, especially charge converting gene delivery systems for treatment of cystic fibrosis (CF) are in focus of research. CF is a monogenic lethal disease that affects around 70 000 patients worldwide. It is manifested by mutations in the CFTR gene encoding a channel in epithelial cell membranes that regulates the liquid volume. The dysfunction of CFTR gene leads to the secretion and accumulation of viscous mucus primarily in respiratory and GI tract. The tenacious mucus enables chronic progressive infection by opportunistic bacteria. This infection and consequently inflammation leading to a vicious cycle is the most common cause of morbidity and mortality in CF patients. However, CFTR gene therapy could be used for the treatment of CF irrespective to mutation. Prüfert et al. developed charge converting NCs as CFTR gene delivery system in order to overcome the mucus barrier which is considered as the main hindrance in CF patients. These NCs containing phosphate substrutures permeate rapidly across the mucus gel layer due to their negative zeta potential. ALP present on the brush border cleaved off the phosphate substrutures which enhanced their transfection on epithelium as positively charged NCs improved their uptake by epithelial cells. They observed a significant transfection of plasmid DNA (pDNA) compared to Lipofectamine 2000 on Caco-2 cells and HEK-293 cells. In another study, transfection efficiency of model pDNA (pcDNA3-EGFP) was improved by developing zeta potential changing lipid-based NCs. For this, lipophilicity of pDNA was enhanced via hydrophobic ion pairing using various counterions in order to incorporate into the lipid-based NCs containing zeta potential changing surfactants. These NCs showed 4.3-fold improved permeation through CF sputum. On reaching to the underlying epithelium, ALP cleaved off the phosphate groups followed by change in their zeta potential. After enzymatic cleavage, a 7.2-fold improved transfection efficiency of pDNA on HEK-293 cells compared to naked DNA was observed. Therefore, ALP triggered zeta potential changing NCs represent a promising gene delivery system for the treatment of CF patients as illustrated in Figure 13.

Ocular surface is protected by two mucosal epithelia namely the conjunctiva and cornea. These mucosal epithelia are directly contact with the tear film and serve as barriers for the administration of topical drugs which limits the efficient infiltration of DNA and drugs into the eye. Therefore, these barriers must be overcome for an effective mucosal drug delivery to the eye. In this regard, charge reversal strategy using ALP is a promising tool for ocular drug delivery due to presence of previous reports indicating the activity of ALP on ocular area. Even though this strategy has not been so far applied to deliver drug molecules or genes across the mucosal barrier of ocular surfaces, it deserves more investigation for further research.

An overview about so far developed charge-changing mucosal DDS triggered by ALP is provided in Table 5.
Furthermore, charge converting delivery systems play a key role in the targeting of tumor cells as positively charged NCs could be efficiently taken up by these cells via electrostatic interactions. As ALP changes the surface charge of the phosphorylated NCs after cleavage of the phosphate moieties, resulting cationic NCs can be retained by tumor cells. Perttu and co-workers developed charge converting liposomes containing inverse phosphocholine lipids with a phosphate moiety. On the tumor cell surface, ALP cleaved off the phosphate from phosphocholine lipid in order to generate a cationic surface of the liposome being a useful tool for cytoplasmic delivery of the encapsulated drugs. In another study, authors designed polyion micelles (Pt-PIC) using a cisplatin containing cationic polymer (P(DSP-DAEP)) and methoxy poly(ethylene glycol)-block-poly(L-phosphotyrosine) (mPEG-b-PpY) to enable the selective delivery of the chemotherapeutic drug cisplatin into ALP-overexpressing cancer cells.

Upon incubation with ALP, (Pt-PIC) exhibited a zeta potential change from $-71$ to $-21$ mV. Correspondingly, cellular uptake of (Pt-PIC) was similar to P(DSP-DAEP) in ALP-expressing SAOS-2 cells unlike ALP-omitting U-2OS cells, indicating a cell-selective uptake of cisplatin depending on ALP expression (Figure 14).

Cell-penetrating peptides (CPPs) are on medical research for nearly 30 years and have the ability to move across cell membranes as well as to carry therapeutics into the various type of cells by an energy-independent mechanism. In order to express cell-penetrating properties, peptides should have certain structural features including a positive charge that is mostly provided by arginine and lysine subunits and hydrophobic domains. However, a cationic surface is not favorable for DDS due to undesired coverage of the carrier before reaching the target site as mentioned above. Therefore, ALP-responsive charge reversal DDS were utilized together with CPPs to mask the cationic charge of the peptides by phosphate moieties. For instance, liposomes were prepared with a phosphorylated cell-penetrating lipopeptide (FP-2PT-Chems) in order to control membrane destabilization effect of the peptides by masking them with phosphate groups. Following phosphate cleavage by ALP a shift in zeta potential from $-17$ to $-7.5$ mV was observed for FP-2PT-Chems loaded liposomes. Moreover, an enhanced cellular uptake was reported for FP-2PT-Chems loaded liposomes enabling the delivery of a fluorophore (propidium iodide) near to the nucleus. Unlike FP-2PT-Chems loaded liposomes, control liposomes containing a sulfonated lipo-peptide (FP-2Sul-Chems) were not uptaken by B16F10 cells showing that uptake took place upon removal of phosphate moieties by ALP. Similarly, surface of poly-L-lysine bearing nanoemulsions was covered by polyphosphates through electrostatic interactions to generate charge reversal.

**Figure 13.** Schematic presentation of charge-reversal gene delivery systems for treatment of pulmonary cystic fibrosis.
| Nanocarriers   | Mechanism                                                                 | Name of excipient                                                                 | Mucus permeation | Cellular uptake / transfection efficiency | Functions                                                                 | Refs. |
|---------------|---------------------------------------------------------------------------|-----------------------------------------------------------------------------------|------------------|------------------------------------------|---------------------------------------------------------------------------|-------|
| Nanoparticles | Chemical phosphorylation of the excipients                               | Phosphorylated carboxymethyl cellulose-glucosamine 6-phosphate and polyethylene imine conjugate | –                | 3.3-fold                                  | Promising strategy for pDNA delivery to target cells                      | [202] |
|               | Synthesis of nanoparticles utilizing polyphosphates                      | Polyethylene imine and polyphosphate                                             | 2-fold           | 2.6-fold                                  | Simple preparation of NCs, promising mucosal and gene drug delivery        | [185] |
|               | Surface phosphorylation of nanoparticles                                  | Chitosan–chondroitin sulfate nanoparticles                                         | 2.1-fold         | 2-fold                                    | Promising mucosal and gene drug delivery                                  | [200] |
|               | Phosphorylation via carboximide chemistry                                 | 6-phosphogluconic acid and polyethyleneimine (branched)                           | –                | 1.3-fold                                  | Mucosal pDNA delivery                                                     | [204] |
| Micelles      | Chitosan and steric acid conjugate synthesized via amidation              | Phosphorylated chitosan-stearic acid conjugate                                   | 6-fold           | 3.2-fold                                  | Mucosal delivery of macromolecular compounds                               | [64]  |
| SEDDS         | Flip-flop mechanism                                                      | Phosphorylated serine-oleylamine conjugate                                       | 2-fold           | 2-fold                                    | Overcome the mucosal barrier                                              | [96, 221] |
|               | Janus-headed surfactant                                                  | Phosphorylated 12-amino-dodecanol                                                 | 6.5-fold         | –                                        | Improve mucosal drug delivery                                             | [222] |
|               | Cationic surfactant and phosphate bearing agent incorporated into oily droplets | 1,2-dipalmityl-sn-glycero-3-phosphatid acid sodium (PA)                          | 2.5-fold         | –                                        | Improved mucus permeation and successful tool for absorption of lipophilic DDS | [67]  |
|               | Phosphorylated emulsifier exhibiting PEG linker                          | N,N′-bis(polyoxyethylene)oleylamine bisphosphate (POAP)                          | 3-fold           | –                                        | Phosphorylated compound containing hydrophilic PEG linker are promising tool for developing zeta potential changing NCs for mucosal drug delivery | [218] |
|               | Acyl substitution through covalent amide bond                            | Stearic acid phosphotyrosine amide                                                | 3.6-fold         | –                                        | Synthesized excipient is a promising tool for developing zeta potential changing system | [225] |
|               | Phosphorylation of polymers using phosphorous pentoxides                 | Starch phosphate and hydroxypropyl starch phosphate                              | 2.5-fold and 5.4-fold | –                                        | Strategy for enhancing bioavailability of drugs administered through mucosal route | [193] |
|               | Effect of water movement on mucus permeation                             | Desmopressin loaded oily droplets                                                  | 5-fold           | –                                        | Mucus permeation of NCs is independent from drug release                  | [250] |
| Nanoemulsions | Zwitterions used as auxiliary agent                                       | Phosphorylated tyramine                                                            | 8.8-fold         | 2.4-fold                                  | Alternative to cationic surfactants                                       | [223] |
|               | Impact of PEG-corona on zeta potential changing nanoemulsions            | PEG free nanoemulsions                                                            | 2-fold           | –                                        | Enhanced zeta potential changing behavior of nanoemulsions to improve mucosal drug delivery | [213] |
|               | Phosphorylated surfactant exhibiting PEG spacer                           | Polyoxyethylene (9) norylphenol monophosphate ester (PNPP)                        | –                | Improved                                  | Promising strategy for developing zeta potential changing system         | [104] |
|               | Decoration of NCs with polycationic cell penetrating peptide (CPP) followed by polyphosphate coating | Poly-L-lysine decorated oily droplet coated with phytic acid and triphosphate     | –                | 4-fold                                    | Overcome polycation dilemma                                               | [105] |
nanoemulsions.\textsuperscript{[105]} Upon removal of the surface polyphosphate coating by ALP, zeta potential raised from $-14.1$ to $+4.2$ mV leading to a fourfold enhanced cellular uptake compared with control NCs on Caco-2 cells.

5.2. Self-Assembling Drug Delivery Systems

Being involved in approximately 30\% of both intra- and extracellular biological processes, ALP has been employed as trigger for the generation of self-assembling biomaterials. Previous research focused on the use of phosphatase to activate the biotransformation of fibers, micelles, and hydrogels.\textsuperscript{[262,263]} In particular, the merge of peptide- and gene therapy-based nanotechnologies led to the development of self-assembling drug NCs with both controllable responses to every disease stage and a predictable toxicity.\textsuperscript{[264]}

The likely first phosphatase-induced self-assembling supramolecular hydrogel system was developed by using ALP to dephosphorylate N-(fluorenylmethyloxycarbonyl) (Fmoc) tyrosine phosphate (FpY) to yield a hydrogelaotor (FY).\textsuperscript{[208]} Under basic conditions, this amphiphilic $\alpha$-amino acid transformed into hydrogels. Expanding their previous investigations, Yang et al. developed a phosphatase-triggered self-assembling hydrogel of $\beta$-amino acid derivatives.\textsuperscript{[205]} A chimera of tripeptides consisted of $\beta^1$-homo-phenylglycine dipeptide and tyrosine phosphate was designed as a hydrogel precursor. The self-assembling hydrogels established from $\beta$-amino acid derivatives exhibited higher stability than that from $\alpha$-amino acid derivatives.

Li et al. explored a different type of hydrogelators composed of nucleobases and amino acids.\textsuperscript{[226]} A nucleobase like adenine, guanine, thymine, or cytosine was connected to a dipeptide L-Phe-L-Phe (FF), and subsequently attached to tyrosine phosphate to yield a precursor. Upon triggered by ALP under physiological pH, the nucleopeptide precursors transformed into hydrogelators that reconstructed into nanofibers and form hydrogels at low concentration. Besides biocompatibility, these hydrogelators exhibited excellent resistance to enzyme digestion, in detail proteinase K, a protease that can break up a broad spectrum of peptidic substrates. Showing negligible inhibitory capability on the cellular migration using a wound-healing assay, furthermore, these nanofibers and hydrogels also showed great

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**Figure 14.** ALP-activated cell-selective delivery of chemotherapeutic drug upon cell internalization. Adapted with permission.\textsuperscript{[224]} Copyright 2017, Royal Society of Chemistry.
potential as biomaterials for maintaining cell–matrix interaction. Similarly, other studies demonstrated that supramolecular nanofibers formed by phosphatase-induced self-assembly of dipeptide derivative or N-capped d-tetrapeptide derivative could accumulate in the pericellular as well as intracellular space and inhibit cancer cell growth.\cite{206,227,228}

In order to design a multi-functional hydrogel delivery system, a precursor based on taxol was investigated.\cite{229} The precursor consisted of a self-assembling pentapeptidic segment, a phosphatase-cleavable tyrosine phosphate residue, a succinic acid linker, and a taxol unit. With the aid of ALP, dephosphorylated precursor as a hydrogelator could self-assemble into β-sheet-like structured nanofibers that were of high density and transformed into sheet-like matrices hydrogel. Preliminary findings also demonstrated that the supramolecular hydrogel could release the substrate in a rate-controlled manner. Other in situ forming supramolecular nanofibers based on taxol-phosphorylated peptide conjugates had been developed.\cite{230} The phosphorylated dipeptide (phenylalanine-phosphoTyrosine) or \(\text{DFP}_{\text{Y}}\) (the D-configuration of FpY) was designed by Fmoc solid-phase peptide synthesis. The succinimide activated taxol was conjugated with FpY or \(\text{DFP}_{\text{Y}}\) dipeptide to afford the \(\text{L- or D-precursor. Upon exposed to phosphatase from HeLa and HepG2 cancer cells, the precursor co-assembled into nanofibers forming 3D hydrogel networks within or around cellular environment. These hydrogels subsequently disturbed tubulin arrangements and induced the apoptosis of cancer cells. In addition, their observations also showed that D-hydrogel exhibited better mechanical strength than L-hydrogel.

Similarly, a precursor Nap-Phe-Phe-Tyr(H\(\text{PO}_{4}\)\(\text{J})\)-OH (1P) was decorated with an anticancer prodrug etoposide phosphate (EP).\cite{210} When being dephosphorylated by ALP, the resulting hydrogelator coassembled with etoposide into nanofibers that showed a sustained release of etoposide leading to controlled inhibitory effects on cancer cells. In tumor-bearing mice treated with 1P+EP, the tumor growth was further inhibited and body weight loss was reduced in comparison with EP-treated mice. As overdose of etoposide may cause immunosuppression in patients, this strategy enabled the controlled release of drug from nanofibers to enhance its antitumor effect without noticeable adverse effects. Li et al. compared the ALP-induced hydrogelation of L- and D-peptidic hydrogelators.\cite{68} The authors showed that the chirality of the precursors played a minor role on the hydrogelation triggered by enzymatic phosphate cleavage of tyrosine phosphate moiety. In addition, the conjugate between a therapeutic agent (e.g., anticancer drug such as taxol) or a fluorophore dye (e.g., 4-nitro-2,1,3-cyclooctene (TCO) generating the inactive TCO-Dox prodrug.\cite{185} The trans-yl)phenyl) acetic acid), which was conjugated to lysine. The anticancer drug doxorubicin (Dox) was coupled with a trans-cyclooctene (TCO) generating the inactive TCO-Dox prodrug. When Tz and TCO moieties on NapK(Tz)pYF and TCO-Dox get into contact, they react via inverse-electron-demand Diels–Alder mechanism,\cite{169} and trigger the decaging reaction releasing Dox. For an in vivo study on tumor-bearing mice, NapK(Tz)pYF at 50 mg kg\(^{-1}\) was i.v. administered 2 h prior to the i.v. injection of 30 mg kg\(^{-1}\) of TCO-Dox. Results showed that mice treated with NapK(Tz)pYF + TCO-Dox for 14 d exhibited tumor shrinkage that was similar to mice treated with native Dox, whereas their body weights remained steady and histological analysis of major organs was similar to mice treated with saline. Further-
more, single-photon emission computed tomography/computed tomography imaging and UPLC-MS/MS analysis confirmed the selective accumulation of NapK(Tz)pYF and decaged Dox in tumors over liver and plasma. This strategy could also be employed to deliver other toxins in close proximity to cell organelles with high therapeutic efficacy and significantly reduced adverse drug reactions.

Besides targeting tumors, it is demonstrated that enzymatic induced self-assembly of peptidic amphiphiles can mediate bacterial cell death by the formation of intracellular nanofibers. Self-assemblies of such materials can be activated by the attachment of an enzymatic responsive functional substructure.\textsuperscript{69} ALP has been utilized to trigger the self-assembly of aromatic peptidic derivatives by converting phosphate-containing precursors into self-assembling elements. Hughes et al. developed a derivative of Fmoc protected self-assembling dipetide amphiphiles as ALP-responsive precursors. It has been revealed that the addition of inosine to culturing media of \textit{E. coli} enhance the overexpression of ALP. Nonassembling precursors after ALP treatment can transform to self-assembling molecules. These self-assembling amphiphiles exhibit an antimicrobial response within the cultured \textit{E. coli}.\textsuperscript{270} Therefore, the strategy of self-assembling DDS by ALP boosts the therapeutic efficacy of drugs.\textsuperscript{244} In another study, dexamethasone sodium phosphate conjugated with the precursor Nap-Phe-Phe-Tyr-(H$_2$PO$_3$)-OH could coassemble after ALP treatment to form nanofibers. These nanofibers exhibited anti-inflammatory response because of higher cellular uptake and increasing retention time of dexamethasone sodium on the cells.\textsuperscript{271} Yang et al. observed the cellular response by using intracellular hydrogelation of small molecules in \textit{E. coli}.\textsuperscript{272} ALP present in \textit{E. coli} was originated to catalyze the hydrogelator formation inside the bacterial cell.

Not only being able to dephosphorylate peptidic derivatives, ALP can also efficiently cleave phosphate groups from polymers or block copolymers. However, ALP triggered self-assembled hydrogel of nonpeptidic materials is relatively rare. For example, Phillips et al. reported the design of poly(oligoethylene glycol methacrylate)-based polymeric systems whose cloud points were effectively controlled by dephosphorylation.\textsuperscript{227} By adjusting the density of phosphate moieties on the polymeric backbone, the system could generate an isothermal transition and undergo coil–globule transformation at a constant temperature. In a recent investigation, Fries et al. used a carbohydrate amphiphile N-(fluorenlymethoxycarbonyl)-glucosamine-6-phosphate to investigate the phosphatase concentration dependence on self-assembling effect via ALP expressing cell lines.\textsuperscript{238} Their results showed the carbohydrate-based precursor upon activation by high ALP activity of osteosarcoma cells could form a virulent nanonet or hydrogel “cage” adjacent to cancer cells leading to the cell apoptosis.

Following the concept using $\alpha$-amino acid derivative as hydrogelator, a reversible sol–gel system was developed by using the kinase/phosphatase switch.\textsuperscript{198} The research described a synthesized pentapeptidic hydrogelator, namely Nap-FFGEY, which was capable to coassemble into nanofibers of various sizes in gels. Upon treated with kinases and ATP, the self-assembled hydrogel was disrupted due to the change at tyrosine moiety of Nap-FFGEY into a hydrophilic molecule Nap-FFGEY-P(O)(OH)$_2$. Vice versa, when phosphatase was added, the system underwent a phase transition generating uniform nanotubes and forming a 3D hydrogel network. Similar to the approach of self-assembling hydrogels based on small molecules, a reversible kinase/phosphatase trigger was prepared in order to regulate the assembly and disassembly of peptidic amphiphile nanostructures.\textsuperscript{239} A substrate recognition sequence characteristic for protein kinase A was combined into the peptide amphiphile molecule. Upon contact with kinase, peptidic amphiphiles were phosphorylated which disrupted intermolecular $\beta$-sheet formation of supramolecular nanofibers and led to the dissociation of the hydrogel network. Vice versa, phosphatase-induced cleavage of phosphate moiety on serine molecule resulted in the reformation of filamentous nanostructures in the hydrogel. In a notable research, Dong et al. designed fluorescein (FITC)-based precursor, namely FK(FITC)pFFYp (Phenylalanine-Lysine FITC-Phenylalanine-Phenylalanine-phosphoTyrosine),\textsuperscript{240} The phosphatase-responsive precursor could be converted into hydrogelator, namely FK(FITC)FFY, which assembled into nanofibers to form hydrogel and quenched the fluorescence emission. This sol-gel transformation with fluorescence “turn-off” was used as a potential methodology to evaluate the ALP activity in vitro and in living cells.

Recently, significant development of smart materials has been emerged to the investigation of multifunctional DDS. Many delivery systems have been tailored to respond to not only internal but also external stimuli such as catalytic activity of enzymes, temperature, light, or magnet fields. Caponi et al. combined enzyme-responsive self-assembly properties of amino acid derivative FpY (Phenylalanine-phosphoTyrosine) with thermal-responsive characteristics of poly(2-isopropyl-2-oxazoline) (PiPrOx) in order to achieve a dual-responsive system.\textsuperscript{231} Click reaction was conducted to synthesize FpY–PiPrOx-OH polymer bioconjugate (Figure 15). The phosphorylated conjugate formed weak self-assembling structures due to weak interactions between FpY residues. Dephosphorylation reaction induced by ALP rendered a more hydrophobic end group driving the self-assembly into micelles as well as leading to the decrease of the lower critical solution temperature (LCST) by close to physiological temperature. Above the LCST, self-assembling micelles collapsed around the FY core into well-defined mesoglobules, whereas the former weak self-assembling structures exhibited mixed aggregation. Similarly, peptidic precursors were developed as chemotheraphy via targeting downregulation in cancer cells.\textsuperscript{241} Precursors contained a phosphatase-induced cleavage of phosphate moiety on serine and led to the dissociation of the hydrogel network. Vice versa, phosphatase-induced cleavage of phosphate moiety on serine molecule resulted in the reformation of filamentous nanostructures in the hydrogel. In a notable research, Dong et al. designed fluorescein (FITC)-based precursor, namely FK(FITC)pFFYp (Phenylalanine-Lysine FITC-Phenylalanine-Phenylalanine-phosphoTyrosine),\textsuperscript{240} The phosphatase-responsive precursor could be converted into hydrogelator, namely FK(FITC)FFY, which assembled into nanofibers to form hydrogel and quenched the fluorescence emission. This sol-gel transformation with fluorescence “turn-off” was used as a potential methodology to evaluate the ALP activity in vitro and in living cells.

5.3. Aggregating Drug Delivery Systems

Dephosphorylation of polymers causes a change in the hydrophilic character and can subsequently trigger aggregation or disaggregation. Du et al. studied the ability of D-phosphotyrosine ($^{p}$pY) decorated magnetic nanoparticles (Fe$_3$O$_4$-$^{p}$Tyr) to aggregate and to adhere on cancer cells via phosphate cleavage
Figure 15. A) Cationic ring-opening polymerization used to obtain propargyl-PiPrOx-OH (3); B) coupling reaction of Fmoc-pY with 11-azido-3,6,9-trioxaundecan-1-amine utilizing DIC and NHS to obtain Fmoc-pY-azide; C) click reaction used to obtain Fmoc-pY-PiPrOx-OH (1). Adapted with permission.\textsuperscript{[211]}Copyright 2011, Royal Society of Chemistry.

triggered by ALP on the membrane of cancer cells.\textsuperscript{[242]} When replacing the $^1$pY by L-phosphotyrosine ($^2$pY), they found that $^2$pY decorated nanoparticles (NP@$^2$pYs) were dephosphorylated eight times faster than NP@$^1$pYs.\textsuperscript{[243]} Thus, the conversion into NP@$^1$pYs happened mostly in culture medium that inhibited the aggregation of NP@$^1$pYs on the cell surface. Meanwhile, NP@$^2$pYs which were primarily dephosphorylated on the cell surface could form aggregates and adhere specifically to cancer cells leading to cell death (Figure 16). Wang et al. fabricated oligomer precursors based on the attachment of redox modulator triphenylphosphonium to the $\epsilon$-amine of lysine moiety on phosphorylated tetrapeptide.\textsuperscript{[244]} Upon cleavage of phosphate group by ALP, resulting structures accumulated to form nanoassemblies via noncovalent bonding. These redox assemblies are internalized mainly by a caveolae/raft-dependent endocytosis pathway selectively targeted mitochondria killing cancer cells.

Recently, Yang et al. designed gold nanoparticles (AuNPs) with a phosphatase-cleavable peptide sequence (namely CREKA-YPFFK naphthalimide (Nph)).\textsuperscript{[245]} The CREKA-YPFFK(Nph) peptide consisted of a pentapeptide structure as a ligand targeted fibronectin, the YPFFK(Nph) as an ALP-responsive residue, and a self-assembling precursor labeled with a green fluorescence dye. When exposed to ALP, the cleavage of phosphate triggered the aggregation of gold nanoparticles through intermolecular hydrogen interactions and subsequently increased the retention of gold nanoparticles in tumors. Following the strategy describe above, phosphotyrosine (pY) modified gold nanoparticles were designed for the delivery of doxorubicin.\textsuperscript{[246]} Zhang et al. fabricated a ternary cyclodextrin-liposome supramolecular nanoassembly as a therapy for acute liver injury. $\beta$-Cyclodextrin was modified with phosphotyramine, an enzymatically cleavable functional group, and loaded with the hepatoprotective drug sili-
binin (SLB31) via host-guest inclusion. Upon ALP triggering, ternary nanoassemblies formed by the interaction of the SLB31 complex with the amphiphilic phosphatidylcholine showed targeted aggregation and drug release in the liver of mice.

To specifically deliver drug at epithelial surface of GI tract, Le-Vinh et al. have recently designed phosphatase-responsive size-shifting SLNs decorated with a phosphate ester containing surfactant and a fatty amine (i.e., octadecylamine). The phosphate ester containing surfactant provided a negative charge shell, which enhanced the mucus penetration of the SLN to reach the epithelial cell surface. Once dephosphorylated by cell-membrane bound ALP, SLNs lose their negative charges and expose the positively charged octadecylamine residues, thus leading to the aggregation of SLNs above the cellular surface as shown in Figure 17.

5.4. Disaggregating Drug Delivery Systems

Besides triggered the self-assembling and the aggregation of drug delivery systems, ALP also induced the disaggregation of nanocarriers. It can serve as a promising approach for the specific design of drug delivery systems. For example, aggregates were formed as a result of non-covalent interactions between a hydrophilic block copolymer of PEG derivative (PEG-b-PLKC) and ATP. Upon treatment with ALP, ATP bearing multiple negative charge phosphate functional groups was transformed into single-charged phosphate moiety and a neutral adenosine residue leading to the dissociation of PEG-b-PLKC-ATP complex and the disassembly of previously formed aggregates (Figure 18).

Wang et al. fabricated a supramolecular amphiphilic assembly relied on the host–guest complexes between calixarene (AC4AH) and ATP. Via the hydrolysis of ATP catalyzed by phosphatase, the small micelles can be dissipated. Leichner et al. prepared chitosan nanoparticles cross-linked with tripolyphosphate (TPP) as delivery system for β-galactosidase. Being dephosphorylated by ALP on the absorption membrane, the TPP cross-links within the particle network was disrupted leading to the disaggregation of nanoparticles and the release of active ingredient in a controlled manner. As β-galactosidase is highly susceptible to the metabolizing intestinal environment, the incorporation of β-galactosidase into chitosan/TPP nanoparticles could maintain the intact of the enzyme until reaching the target mucosal tissues.

6. ALP Triggered Probes in Diagnosis

ALP is widely expressed in many tissues at various levels. The activity of ALP usually rises in various human diseases such as hepatitis, obstructive jaundice, osteomalacia, and many types of cancer. It is reported that ALP is over-expressed on the membrane of certain cancer cells and is considered as one of the essential biomarkers for ALP-associated cancer diagnosis. Currently, various ALP mediated fluorescent tools such as ALP triggered molecular probes, ALP induced self-assembling systems, ALP induced aggregating systems, or multimode imaging probes for the diagnosis of various diseases and tumors have been under investigation. A brief overview of ALP-related diagnostics is illustrated in Table 6.

ALP triggered fluorescent molecular probes have been utilized to detect ALP activity in vitro and in vivo, and are critically reviewed. Thanks to their high sensitivity and selectivity, and noninvasive properties, they have huge potentials not just in ALP activity determination and ALP inhibitor screening but also...
Phosphate-functionalized solid lipid nanoparticles (SLN) can effectively penetrate mucus gel layer. Upon exposure with ALP at epithelial surface, SLN aggregates were formed due to interparticle electric interactions induced by enzymatic phosphate removal, thus avoiding back diffusion effect. These aggregates remained at the cell surface and slowly released loaded drug. Below: processed confocal images (XZ-maximum projection) of SLN on Caco-2 cell layers covered with purified porcine intestinal mucus showing big SLN aggregates (yellow clusters) allocated on cell layer surface (C1), whereas less and small SLN aggregates were formed in the presence of ALP inhibitors at 0.8% v/v (C2). Adapted with permission.[7] Copyright 2021, Elsevier.

In biological imaging and real-time detection. For example, Liu et al. described an ALP-mediated near infrared (NIR) fluorescent probe to improve the in vivo imaging of ALP in tumor-bearing mouse. The activity of the probe was based on the transfer of intramolecular charge by shielding the hydroxyl group of fluorophore with monophosphate which is cleaved by ALP followed by the turn-on of the fluorescent signal. The advantage of utilizing NIR light is that it can penetrate deeply into the tissue and lessen interference from autofluorescence in living systems. The probe showed a high selectivity for ALP which is considered as the potential application for pathological diagnosis of tumors[277] (Figure 19).

In comparison with small probe molecules, in situ self-assembling or self-aggregating probes can better accumulate at target tissues and yield higher signals, thus are favorable for long-term tissue imaging and cancer diagnostics.[276,278–280]

6.1. Self-Assembling Probes

In order to diagnose the superficial cancers, a real time and non-invasive imaging technique based on the ALP activatable probe to enhance the photoacoustic (PA) imaging of tumors was used by Wu et al.[276] They designed an ALP activatable NIR-PA probe that upon dephosphorylation by ALP self-assembled into nanostructures with improved PA signals for tumor imaging as illustrated in Figure 20. In brief, a near-infrared (NIR) probe IR775-Phe-Phe-Tyr(H$_2$PO$_3$)-OH (1P) was rationally fabricated by conjugating the NIR dye IR775 to a peptidic structure for ALP-responsive self-assembly. Under the catalysis of ALP on tumor cell surfaces, hydrophilic 1P was dephosphorylated into more hydrophobic 1 that could penetrate into tumor cells and self-assembled to form 1-NPs nanoparticles leading to a 6.4-fold enhancement in photoacoustic signal compared to 1P molecule. NIR fluorescence
image-guided surgery has been available for investigation in clinical trials.\textsuperscript{[281]}

In another study, a combination of a fluorescent staining agent, a quinazolinone derivative based on ELF97, and D-tripeptides (\textsuperscript{\textregistered}Phe-\textsuperscript{\textregistered}Phe-\textsuperscript{\textregistered}Tyr) was designed as imaging probes.\textsuperscript{[282]} In order to enhance the self-assembling capability of D-tripeptides, carboxylic acid group at the C terminus was replaced by a methoxycarbonyl (1a) or (methylamino)carbonyl (1b) group (Figure 21). Upon enzymatic cleavage by ALP, the probes reduced their hydrophilicity and self-assembled resulting in the conversion of the weakly blue fluorescent of ELF97 to bright-green fluorescent. Interestingly, transmission electron microscopic images showed that self-assembled probes with 1a substructure contained sparse nanofibers while self-assembled probes with 1b substructure produced aggregates.

Integration of a fluorophore with peptidic supramolecular materials can further broaden the realm of phosphatase-instructed self-assembly of nanofibers and hydrogels. The turn “on” or “off” of a fluorescence can be controlled in order to use as a biomarker sensing. For example, a research aimed to design tumor-specific phosphatase-triggered indocyanine green (ICG)-doped nanofibers for cancer theranostics has been reported by Huang et al.\textsuperscript{[233]} The dephosphorylation of tyrosine phosphate residue on the peptide precursor resulted in the intermolecular head-to-tail arrangement between peptide and ICG into nanofibers. The entrapment of ICG molecules into nanofibers induced the red shift and significantly higher near-infrared absorption of ICG. Furthermore, Zhou and co-workers designed a phosphorylated and 4-nitro-2,1,3-benzoxadiazole coupled D-peptide (pNDP1) as an imaging probe for ALP.\textsuperscript{[227]} Being dephosphorylated by ALP, pNDP1 transformed into NDP1, which self-assembled to produce non-diffusive and fluorescent nanofibrils of NDP1 nearby for imaging the activities of phosphatase on living cells. Similarly, an ALP-sensitive fluorescent probe (TPEPy-pY) was constructed for the detection of bacterial ALP.\textsuperscript{[235]} TPEPy-pY consisted of a self-assembling peptide derivative D-pY in conjugation with aggregation-induced emission luminogens. Upon dephosphorylated by ALP on the surface of E. coli, TPEPy-pY reduced its hydrophilicity and caused the re-assembly of the probe. The resulting fibrous structure aggregated on surface of the bacteria and triggered fluorescence.

In recent years, the concept of multimode imaging probes has been introduced in the field of diagnostics and biomedical research.\textsuperscript{[283]} Multimodality imaging probes exhibiting a synergistic combination of NIR fluorescence and magnetic resonance imaging (MRI) play a key role in the diagnosis of malignant tumors. Various NIR fluorescence and MRI bimodal imaging probes have been reported for molecular imaging.\textsuperscript{[284,285]} How-

Figure 18. Self-assembled aggregates of PEG-b-PLKC-ATP amphiphile. Upon dephosphorylation of ATP induced by phosphatase, the system was disaggregated. Adapted with permission.\textsuperscript{[248]} Copyright 2010, Wiley-VCH.
Table 6. Examples of ALP induced probes and their diagnostic applications.

| Mechanism               | Diagnostic agents/probes                                                                 | Substrate                          | Application                                                                 | Refs. |
|-------------------------|------------------------------------------------------------------------------------------|------------------------------------|-----------------------------------------------------------------------------|-------|
| Self-assembly           | A fluorophore, 4-nitro-2,1,3-benzoazidazole and a phosphorous ester, tyrosine phosphate | Tyrosine phosphate residue         | Explore supramolecular imaging inside the cells                              | [296] |
|                         | A peptide derivative, NBD-GfpFss-ERGD                                                    | Phosphate moiety                   | For liver cancer diagnostics                                                | [275] |
|                         | A mitochondria-targeted near-infrared (NIR) activatable fluorescent/ photoacoustic probe | Phosphate ester moiety             | For biomodal imaging of prostate cancer                                      | [297] |
|                         | A hydrogelator, Nap-FFYp-EDA-DOTA(Gd)                                                    | Phosphate moiety of the probe       | Enhanced magnetic imaging of the tumors, imaging in vivo enzyme activity     | [298] |
|                         | Enzyme induced self-assembly based monomer-eximer transition of low molecular concentration coumarin dye | L-Phosphotyrosine residue           | Supramolecular assemblies with enhanced imaging performance                  | [299] |
| Aggregation             | Hybridized electrochemical biosensor                                                     | Intracellular pyrophosphate         | Wireless biosensors diagnose the cancer by investigating the interaction between cells and modified surfaces | [300] |
|                         | Nitrogen doped carbon dots                                                              | Pyrophosphate                       | Detection of ALP activity for clinical diagnostics                           | [301] |
| Chemiluminescence      | Enzyme-activated chemiluminescence nanoprobe                                             | Tyrosine phosphate residue          | Selective tumor imaging                                                      | [288] |
|                         | An electrogenerated chemiluminescence biosensor, concanavalin A coated gold nanoparticles (Au NPs) nanoprobe | (3-[2-Spiroadamatane]-4-methoxy-4-[3-phosphoryloxy]-phenyl-1,2-dioxetane)dioxetane | Diagnostic tool for drug induced liver imaging                              | [302] |
|                         | An electrochemiluminescence system, coreaction of CdSe NCs and triethylamine             | Phenyl phosphate disodium           | Evaluate the expression of glycans on cell surfaces                           | [303] |
|                         | Activatable molecular probes                                                             | Phenyl phosphate salt               | Complex biological matrix detection                                          | [304] |
|                         |                                                                                            |                                    | Deep tissue in vivo imaging                                                  | [305] |

Figure 19. Near-infrared (NIR) fluorescent probe cleaved by ALP resulting in turn-on fluorescent signals. Adapted with permission.[277] Copyright 2017, Elsevier.

However, these bimodal probes exhibit “always-on” signals irrespective of interactions with tumor tissues. Therefore, the development of small-molecule activatable probes having the ability to diffuse deeply into tumor tissues and simultaneously “switching-on” NIR/MRI signals upon interaction with tumor cells are in focus. Yan et al. developed activatable NIR fluorescence/MRI bimodal probes by ALP triggered fluorescent reaction and by an in situ self-assembly approach for in vivo imaging. Endogenous ALP expressed on the cell membrane can activate the bimodal probe (PCyFF-Gd) resulting in the production of membrane localized self-assembled NCs. These NCs simultaneously enhanced NIR fluorescence more than 70-fold and relativity $\approx 2.3$-fold due to the dephosphorylation followed by self-assembly of the bimodal probe by ALP. This bimodal probe enables high-resolution imaging in live tumor cells on the one hand and facilitates efficient imaging-guided surgical resection of tumor tissues in mice on the other hand. The ALP-associated bimodal imaging probes with synergistic combination of NIR fluorogenic and MRI might be a useful tool in cancer theranostics.[286]

In contrast to fluorescence, chemiluminescence that does not require light excitation has a greater signal-to-noise ratio, thus can be utilized as an alternative to monitor an intracellular bioprocess or the enzyme activity. By coincubation of the chemiluminescence agent and the precursor Fmoc-Phe-Phe-Tyr(H2PO3)-OH (1P) with ALP, Hai et al. could employ chemiluminescence to directly track the synchronized hydrogelation process of ALP-triggered self-assembled hydrogelator 1P.[236] In addition, when incorporating an anticancer drug (e.g., doxorubicin) into the system, the hydrogelation process was still able to visualize by chemiluminescence. Moreover, Fan et al. designed an ALP triggered chemiluminescence platform in order to diagnose liver cancer on the one hand and its treatment without using an external light source on the other hand. They utilized 3-[2-spiroadamatane]-4-methoxy-4-[3-phosphoryloxy]-phenyl-1,2-dioxetane dioxetane as chemiluminescence substrate of ALP and mesoporous silica as carrier system. These NCs were loaded with (4-carboxyphenyl) porphyrin.
Figure 20. ALP in tumor converts 1P (IR775-Phe-Phe-Tyr(H₂PO₃)-OH) to 1 (IR775-Phe-Phe-Tyr-OH) which subsequently assembles into 1-NPs nanoparticles inside the tumor enhancing photoacoustic imaging of tumors. Adapted with permission. [276] Copyright 2018, American Chemical Society.

Figure 21. The conjugation of a D-tripeptide to the quinazolinone derivative ELF97 to generate a cell-compatible and nondiffusive probe for selective cell imaging. Adapted with permission. [282] Copyright 2019, Wiley-VCH.

and further integrated with β-cyclodextrin. ALP expressed on the liver cancer cells hydrolyzed these NCs result in the excitation of (4-carboxyphenyl) porphyrin through chemiluminescence resonance energy transfer. Chemiluminescence excited NCs provides a promising platform for imaging of the liver tumors on the one hand and treatment of these tumors in the deep tissues on the other hand. [287]

6.2. Self-Aggregating Probes

Aggregation-induced emission (AIE) is a photophysical phenomenon in which nonemissive molecules in dissolved state become emissive by aggregate formation. [288] In dissolved state, probe molecules can easily distribute in the body and penetrate into tissues, while in aggregation state triggered by fac-
tors like enzymes such as phosphatases or radiation, the probes can accumulate and be retained longer at the target tissues. Thanks to high imaging resolution and contrast, this phenomenon is widely studied in live cell and tissue imaging. Ji and co-workers utilized the strategy of enzyme-induced self-assembly to develop a fluorescence and reactive oxygen species generation capability activable probe to enhance the detection of cancer cells. They synthesized a theranostic precursor TPE-Py-FpYGpYP (TPE-Py-Phenylalanine-phosphoTyrosine-Glycine-phosphoTyrosine-Glycine-phosphoTyrosine) that contains an AIE lumogen (TPE-Py, a tetraphenylethene-substituted pyridinium salt) and tyrosine phosphate (Figure 22A). Overexpressed ALP on cancer cells can hydrolyze the phosphate substructure resulting in the formation of self-assembly of the precursor. The self-assembly of the probe inhibits the phenyl ring rotation which would reveal the extensive fluorescence emission. Cellular studies demonstrate that the TPE-Py-FpYGpYP precursor has the capability to differentiate between normal cells and cancer cells based on the overexpression of ALP on cancer cells. Also based on the specific AIE effect, Zhang et al. developed a cationic fluorescent probe bearing a phospho moiety, namely TPEQN-P for detecting and monitoring ALP activity with high sensitivity (Figure 22B). After being dephosphorylated, the water-soluble and nonemissive TPEQN-P converts to the water-insoluble and highly emissive TPEQN. With the same strategy, Liu et al. developed an ALP responsive fluorophore, namely HTQA that is water soluble and in fluorescence "off" state thanks to the shielding of phenolic hydroxyl group with a phosphate group (Figure 22C). Upon exposure to ALP, phosphate was cleaved off generating the strictly water-insoluble HTQA that emitted strong fluorescence with excitation and emission wavelengths at 410 and 550 nm, respectively.

Carbon quantum dots (CQDs) have been considered a suitable alternative to the metal-based semiconductor dots because of their ease in synthesis, low cost, highly biocompatible, fluorescence imaging, low toxicity, and robust photo responsiveness. However, light is a tunable stimulus that interact with CQDs produces fascinating responses that are appropriate for photo-mediated applications. CQDs are suitable candidates in diagnostics when they are combined with photodynamic/photothermal moieties. Tang et al. reported that ALP triggered CQDs nanoprobes improve the targeting of optical biosensors via specific host–guest recognition. They functionalized CQDs with 3-amino-phenylboronic acid and then modified with hydroxypropyl β-cyclodextrins to produce β-cyclodextrins CQD nanoprobes. p-Nitrophenol phosphate disodium salt was employed as a substrate of ALP. After enzymatic cleavage of the phosphate group, p-nitrophenol was produced which could be entered in the cavity of β-cyclodextrins CQD nanoprobes through their host–guest recognition mechanism. Photoinduced electron transfer mechanism between p-nitrophenol and nanoprobe was carried out to reduce the fluorescence of the probe. Choi et al. developed CQDs decorated with dodecane/sulfobetaine group with a Cu²⁺ ion for the detection of cancer cells as these CQDs could permeate into the cell membrane by hydrophobic interactions. They utilized fluorescence on-off-off system in order to target the membrane and nucleus of the cancer cells. Therefore, the introduction of pyrophosphate, a substrate for ALP, redisperse the CQDs by Cu²⁺ ion binding with a stronger binding constant. ALP could cleave off the pyrophosphate to phosphate result in the destruction of pyrophosphate-Cu²⁺ ion complexes. The release Cu²⁺ ion reaggregate the CQDs. As the concentration of pyrophosphate and ALP were different in normal and in cancer cells, therefore, this strategy could selectively and effectively distinguish between normal and cancer cells.

7. Conclusions and Future Perspectives

Since the 1960s, ALP has been a reliable partner for drug delivery and diagnosis. Upon exposure to ALP, phosphate groups are cleaved off from phosphate prodrugs inducing changes in their membrane partitioning properties. These changes do not only increase drug absorption at GI membranes but can also promote site-specific delivery of drugs to tumor cells, and minimize toxicity. When phosphate substructures are removed from their surface, ALP-triggered systems can change their surface charges, morphology, aggregation state, or optical properties leading to more efficient and controllable mucosal drug delivery, targeted drug delivery, tissue and organ imaging, cellular uptake, and gene transfection.

Tailored excipients/probes as substrates of ALP are likely the key in developing new ALP-triggered systems. Apart from direct phosphorylation of polysaccharides, lipids, amino acids and peptides are among preferred bricks for building tailored excipients as they are on the one hand biocompatible and biodegradable materials, and on the other hand somehow mimic the structures of endogenous substrates. Despite numerous triggering pathways by ALP have been reported as presented in this review, we believe more interesting and secure mechanisms are awaiting to be discovered as more knowledge on the physiological roles of ALP and pathogenesis of ALP-related diseases is elucidated. Besides, the discovery of ALP-specific inhibitors is also a challenging but interesting journey. The molecules themselves can be used as therapeutic compounds for some diseases induced by the dysfunction of ALP, e.g., at the intestine or in bone formation, but could also be incorporated into ALP-triggered systems to navigate or modify the performance of the systems.

Despite the bright sides, opportunity always comes with challenges. In view of targeted drug delivery, prodrugs and NCs function with phosphate moieties may be prematurely triggered by phosphatases that are expressed on some tissues surrounding the target tissue, or are present in blood or GI fluid at low levels. This may lead to the deterioration of the designed systems, and may cause adverse effects. To avoid this phenomenon, a so-called “2-factor authentication” or “multifactor authentication” system can be applied. ALP triggering would play as the first or the final authentication step, whereas the other authentication factor could be triggered by another enzyme which is co-present with ALP at the target tissue, or a special condition like pH or temperature at the target tissue. Again, this strategy would need the design of tailored ALP substrates that respond to ALP and another factor, or the combination of two or more enzyme substrates in a system to achieve the specific effect only at the target tissue. In this direction, dual-activated fluorescent probes were already developed for detection of phosphatases utilizing an initial light-activation followed by an enzymatic phosphate cleavage leading to the fluorescent formation on the desired site.
Figure 22. Synthetic pathway of ALP responsive aggregating fluorescent probes: A) TPE-Py-YpYGpYGpY, adapted with permission[289] Copyright 2018, Royal Society of Chemistry. B) TPEQN-P, adapted with permission[290] Copyright 2018, Royal Society of Chemistry. C) HTPQA, adapted with permission[291] Copyright 2017, Wiley-VCH.
In this strategy, an UV- and phosphatase-labile group is used as “warhead” having the hindered phosphate groups by caging molecules namely, 2-nitrobenzoyloxy group. Upon UV exposure caging molecules are cleaved off from the ligand leading to a site-specific fluorescent activation of the system by phosphatases. Indeed, photoactivation strategy is widely applied to the small molecules, proteins, and gene products upon uncaging these compounds by light switch.[307] Therefore, the usage of such UV-labile phosphate caging molecules can be considered as a promising approach for the ALP-activated drug delivery applications to prevent premature degradation of the system by nonspecific interactions. Nevertheless, low tissue penetration of UV light should be considered carefully while designing such systems for therapeutic purposes.[308]

Antimicrobial drug resistance (ADR) is an emerging global public health problem rendering the majority of conventional drug molecules inefficient for effective treatment of infections caused by bacteria, fungi, viruses, or parasites.[309,310] By targeting one of the major ADR mechanisms, i.e., biofilms, NCs are promising DDS due to their nanoscaled size and ease of surface modification enabling their penetration through the biofilm barrier of resistant pathogens. Biofilm formation occurs by the formation of single pathogens to large clusters and the generation of a self-produced matrix around them resisting the penetration of even high antimicrobial drug concentrations as well as immune system components of the host organism into this barrier. Biofilm matrices are composed of extracellular polymeric substances including proteins, polysaccharides, DNA, and other components leading to an overall negative charge of biofilm in the case of most of pathogens.[311] It has been shown that positively charged antibiotics, such as aminoglycosides penetrate through the biofilms slower than negatively charged antibiotics,[311,312] On the contrary, a positive charge is required for an efficient uptake of NCs into the pathogen cell.[313] The production of ALP by most of the resistant bacterial[18,315] as well as fungal[316] was well documented. Therefore, transferring the already established ALP-induced charge conversion technology to ADR research is a promising strategy to develop novel treatment options against microbial biofilms in future.

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Conflict of Interest
The authors declare no conflict of interest.

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