The Pharmaceutical and Chemical Journal, **2021, 8(2):47-56**

Available online www.tpcj.org

Research Article

ISSN: 2349-7092
CODEN(USA): PCJHBA

Research Progress of Phosphorylated Polysaccharides

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Abstract Phosphorylated polysaccharides, as a very important polysaccharide, exist widely in animals, plants and microorganisms. It has a variety of biological activities such as antioxidant, immune, anti-tumor and anti-viral. According to the research, its therapeutic effect is significant, but it is not easy to extract natural phosphorylated polysaccharides, so the artificial synthesis of phosphorylated polysaccharides has become a research hotspot. The reagents used in the phosphorylation of polysaccharides and the biological activities of phosphorylated polysaccharides were reviewed in this paper.

Keywords Polysaccharide, Phosphorylated polysaccharide, Phosphorylation reagent, Biological activity

Introduction

Polysaccharide is a kind of polymeric product of saccharides. For example, starch, glycogen and cellulose are composed of glucose as a basic unit. Among them, starch and glycogen are used as the storage form of glucose in plants and animals, while cellulose serves as the structural component of plant cells. There are also glycosaminoglycans such as hyaluronic acid, chondroitin sulfate and heparin. Glycosaminoglycans are the main components of proteoglycans.

Literature has shown that polysaccharides have antioxidant, inducing cancer cells apoptosis, immunomodulatory, anti-inflammatory, anti-tumor, lowering blood glucose, treating diabetic complications, delaying the *Aspergillus flavus* related gene transcription, treatment of neurons damage and other effects [1-9]. But the water - solubility of polysaccharide is poor, so it can't fully play its role. In order to make polysaccharides widely used, people modified the structure of polysaccharides. These structural modifications can not only change the physicochemical properties of polysaccharides, but also produce new biological activities [10].

We can modify polysaccharides with sulfuration, carboxymethylation, phosphorylation and so on. Sulfated polysaccharides show anti-tumorand antiviral abilities [11]. Carboxymethylated polysaccharides can improve the anticoagulant ability [12]. Phosphorylated polysaccharides can improve antioxidant activity [13] and water solubility [14], etc. Studies have shown that the degree of substitution varies with chemical modification. For example, Chen et al [15] carried out chemical modification of momordicacharantia polysaccharide and found that the degree of substitution of phosphorylated *Momordica charantia* polysaccharide was 0.12, while that of sulfated *Momordica charantia* polysaccharide was 0.45. The improvement of antioxidant capacity is also different with different chemical modification. Cao et al [13] found that phosphorylated derivatives had the highest scavenging levels on DPPH, ferrous ions radical and hydroxyl radical. There are many modification methods for polysaccharides. This paper mainly introduces the phosphorylation methods and biological activities of phosphorylated polysaccharides.

Common phosphorylation reagents of polysaccharides and their advantages and disadvantages

At present, phosphorylation of polysaccharides is usually performed by phosphoric acid, phosphoric anhydride, phosphate or phosphoacyl chloride. However, the use of phosphoric acid or phosphate is often accompanied by acidic or high-temperature reaction conditions, which can easily lead to the degradation of polysaccharides. Phosphoryl chloride can cause crosslinking and other side reactions, making the product more complex. Therefore, it is particularly important to find the appropriate reaction conditions.

Phosphoric acid and phosphoric anhydride

Phosphoric acid and phosphoric anhydride or a mixture of the two are the earliest phosphorylation reagents used. If phosphoric acid is used as phosphorylation reagent and sulfuric acid is used as catalyst, the strong acid condition can easily cause polysaccharide degradation, so it is rarely used. But the method with urea and phosphate can reduce the influence of strong acid. Pan et al [16] put the microcrystalline cellulose and phosphorous acid of the same molar mass into the molten urea, heated at 140 ℃ and flushed with nitrogen. Then the reaction was allowed to work at 150 ℃ for 5 h. The reaction mixture was dissolved in 1M NaOH and precipitated with ethanol. The process was repeated three times. After overnight diafiltration, phosphorylated cellulose is obtained. The triethylamine - phosphoric acid method can also be used to reduce the experimental side effects. Ragab et al [17] put palm frond hemicellulose (PFHC) and rice straw hemicellulose (RSHC) into dimethylformamide respectively and added triethylamine and phosphoric acid to phosphorylate hemicellulose. The phosphorus content of PPFHC and PRSHC was 0.981% and 0.647%, respectively, by molybdenum blue method.

Phosphorus pentoxide, polyphosphoric acid, pyrophosphoric acid and so on can also be used as the phosphorylation reagent of polysaccharides. Bombaldi de Souza et al [18] phosphorylated chitosan using phosphorus oxide, phosphoric acid, triethyl phosphate and hexanol (P_2O_5 / H_3PO_4 /Et_3PO_4 / hexanol method). The results showed that both the hydroxyl and amino groups of chitosan were hydrolyzed and phosphorylated. This method has the advantage of products do not contain dangerous biological compounds. Methanesulfonic acid is also sometimes used as a solvent for phosphorus pentoxide to improve the solubility and reaction rates. This experiment should be carried out at low temperature, but the experimental product is complex, with not only the formation of monoester phosphate, but also the formation of diester phosphate [19].

There's also the classic polyphosphate-tributylamine process. Polyphosphoric acid can be reaction at room temperature. Tributylamine can destroy the intramolecular hydrogen bond of polysaccharides and speed up the reaction. Zhang et al [20] phosphorylate the polysaccharide extracted from *Porphyra haitanensis* with this method. The polysaccharides were placed in formamide solution, added tributylamine and polyphosphoric acid, stirred at room temperature for 24 h, and then water was added, NaOH was added to adjust the pH to 10. Then the solution was evaporated in a vacuum of 37 ℃ and then dialysed with distilled water 24 h to obtain phosphorylated polysaccharides. Different dosage of reagents, reaction time and temperature can produce different reaction product. Hu et al [21] make *Acanthopanax leucorrhizus* water-soluble polysaccharide (ALP) dissolved in formamide, join tributylamine. One group puts 5.0 g polyphosphoric acid, reaction at room temperature for 24 h, get P - ALP1, another group puts 7.0 g polyphosphoric acid, reaction under 30 ℃ 30 h, get P - ALP2.The results show that substitution degree of P-AlP1 (0.19) was smaller than that of P-Alp2 (0.35). Although cyclotripolyphosphate as phosphorus acylation reagent has simple reaction conditions, the cyclotripolyphosphate in the reaction process is easy to degrade and complicate the product, so this method is rarely used. This experiment steps usually add a certain amount of melamine phosphate in water, adjust pH value, add polysaccharide samples, and reaction at room temperature [22].

Phosphate

Phosphates commonly used in the modification of polysaccharide phosphorylation are sodium dihydrogen phosphate, disodium hydrogen phosphate, sodium trimethophosphate (STMP) and sodium tripolyphosphate (STPP), etc. Phosphate is available at a low market price. However, the experimental process of phosphorylation of polysaccharides by phosphate is complex, so controlling the reaction conditions is particularly important.

Sodium dihydrogen phosphate and disodium hydrogen phosphate are often mixed. Different reaction time, temperature, phosphate ratio and total amount can produce different amounts of phosphorus-containing products. Zhang and Wang [23] prepared the starch phosphate ester of Plantain Taro by dry method and found that the optimal reaction condition was 131.6 ℃ for 2.43 h, and the phosphorus yield was 0.58%. The product of this phosphorylation method under weak acid condition is mainly monoester phosphate. Under basic conditions, not only monoesters but also diesters are formed [24].

STMP and STPP are also commonly used phosphorylation reagents. The phosphorus content of the product was different when the polysaccharide was exposed to different reactant ratio, temperature and pH reaction time. Liu et al [12] showed that when the weight ratio was 4/3 (STPP/STMP), the reaction was 3.0 h at 55 ℃, and the pH was 5.0, and the phosphate content of phosphorylated *Sepia esculenta* ink polysaccharide was up to 1.52%. The optimum reaction conditions of different polysaccharides were different. Zhang et al [25] put the polysaccharide from *Trichosanthes* peel into STPP-STMP (the weight ratio was about 6/1), adjusted the pH to 9 with sodium bicarbonate, and reacted at 80℃ for 24h to obtain the phosphorylated polysaccharide. The phosphate content was calculated to be 14.36%. Feng et al [26] put *Radix Cyathulae officinalis* polysaccharide (RCPS) into STPP and STMP complex with a weight ratio of 1:4. After pH was adjusted to 9, 85 ℃ for 7 h, cooled to room temperature, ethanol was added to precipitate at 4 ℃ for 24 h and then lyophilized. The compound is then re-dissolved by water. The distilled water was dialyzed in a dialysis bag with a cut-off molecular weight of 14,000 until the conductivity dropped from 1×10^4 μs/cm to 160 μs/cm. PRCPS was obtained by freeze-drying again, and the phosphate content was 9.52%.

Using sodium trimetaphosphate as cross-linking agent, the polysaccharide is phosphorylated under alkaline conditions, and the pH can be changed to form diester or triester, and the satisfactory degree of substitution can be obtained by changing the amount of phosphate [27]. Liu et al [28] found that the maximum degree of substitution was 0.79 under alkaline conditions and the weight ratio of xylan and STMP was 1/3 at 80℃ for 4 h.

Phosphoryl chloride

Phosphoryl chloride has high reactivity and can be used as phosphotylation reagent to obtain highly substituted phosphorylation products. But phosphorus oxychloride is easily hydrolyzed in the reaction, making the reaction environment highly acidic. Thus, polysaccharides were hydrolyzed and the content of polysaccharide phosphorylated derivatives decreased. Therefore, the alkalinity of pyridine is usually used to neutralize the hydrochloric acid generated in the reaction process into pyridine salt to prevent the hydrolysis of polysaccharides. At the same time, trimethyl phosphate is added to reduce the reactivity of phosphorus oxychloride [24]. However, this experimental method has a strong reaction, low yield and many byproducts, and it is usually accompanied by the generation of polysaccharide diphosphate and triphosphate when forming polysaccharide phosphate monoester, so it has not been widely used.

Xiong et al [29] used phosphorus trichloride as phosphorylation reagent, and the volume ratio of phosphorylation reagent and triethylamine was 1:10. They were added into DMF for reaction and got three different positions of ginseng polysaccharides replaced by phosphate groups. Wang et al [30] used trichlorophosphate-pyridine technology to react at 25 ℃ for 3-4 h, and the degree of substitution was 0.34-0.54. The experiment showed that the reaction increased with the increase of temperature. Cheng et al [31] used phosphorus oxychloride and pyridine to reaction carboxymethylated garlic polysaccharide at 35 ℃ for 30 min. After the reaction was completed, the mixture was cooled to room temperature, and the pH was adjusted to neutral with 1mol/L NaOH solution. Finally, dialysis filtration was carried out, and carboxymethylated -phosphorylated garlic polysaccharide was obtained after drying. The experimental results showed that the degree of phosphate substitution was 0.069. Which may be caused by the insufficient amount of phosphorus oxychloride and the weak substitution ability of phosphate, but the more important reason was that many positions on the sugar ring of garlic polysaccharide using reactants were replaced by carboxymethylation, thus affecting the substitution of phosphate groups.

Other Methods

Solid acid was used instead of strong acid as catalyst. Chen et al [32] found through experiments that phosphotungstic acid catalyst did not cause significant degradation of products. This opens the way to explore the phosphorylation conditions.

3-phosphate propionic acid can also phosphorylate polysaccharides in the presence of catalyst. Wei et al [33] put *Radix Hedysari*polysaccharide into anhydrous DMF, added 3-phosphate propionic acid, and used N, N' dicyclic carbon diimide (DCC) and 4-dimethyl aminopyridine (DMAP) as catalysts to react at room temperature. The experimental results show that the degree of substitution varies with reaction time. At 24h, the highest phosphate content of the product was 66.0%.

All in all, though, polysaccharide phosphorylation methods are improving. However, there are still some problems in the process of polysaccharide phosphorylation, such as degradation of polysaccharide, complex products, low yield and unsatisfactory degree of substitution. Therefore, finding an efficient and simple method for polysaccharide phosphorylation is still the most important.

Biological activity of phosphorylated polysaccharides

Antioxidant Activity

Studies have shown that polysaccharides exhibit excellent antioxidant activity after phosphorylation[34]. *Lactobacillus helveticus* MB2-1 exopolysaccharide was phosphorylated, its structure was changed and its antioxidant capacity was enhanced [35]. Xiong et al [29] phosphorylated ginseng polysaccharide by trichlorophosphate-pyridine method. The antioxidant activity of phosphorylated native ginseng polysaccharide was higher than that of native ginseng polysaccharide. Jiang et al [36] found the *Ulva pertusa*phosphorylation polysaccharides can be obtained through STMP-STPP method, and its antioxidant capacity was enhanced. In addition, the levels of total cholesterol (TC), triglycerides (TG) and low-density lipoprotein (LDL-C) in hyperlipidemic mice were decreased and the levels of high-density lipoprotein (HDL-C) were increased. Thus, the activity of lowering blood lipids in mice was also increased. Liu et al [37] found that after phosphorylation of mannan extracted from yeast cell wall, its anti-lipid peroxidation ability was enhanced. The phosphorylation of glucan in yeast cell wall also showed significant anti-lipid peroxidation effect and scavenging activity of hydroxyl radical and superoxide anion [38].

Most phosphorylated polysaccharides are more antioxidant than other chemically modified derivatives. Chen et al [39] phosphorylated cushaw polysaccharide by the trichlorophosphate -pyridine method and obtained the phosphorylated cushaw polysaccharide with the substitution degree of 0.01 and 0.02, respectively. The results showed that the antioxidant activity of the phosphorylated cushaw polysaccharide with different degrees of substitution was different. They later phosphorylated and sulfurated cushaw polysaccharides. The results showed that the phosphorylated cushaw polysaccharides had a stronger scavenging ability on hydroxyl radicals [40]. Wang et al [41] took guar gum as raw material and phosphorylated it by trichlorophosphate-pyridine method. Antioxidant experiments showed that high degree of substitution of phosphorylated guar gum could enhance the free radical scavenging activity.

Anti-aging Activity

Aging is a common degenerative phenomenon, which can be divided into physiological aging and pathological aging. Many theories of aging have been put forward, such as programmed aging theories, accumulation of damage theories, disposable soma theory, antagonistic pleiotropy theory and price of complexity hypothesis [42]. Although aging is inevitable, we can achieve healthy aging and even delay aging by regulating it with current technology.

In recent years, the anti-aging studies of polysaccharides have emerged one after another, but the anti-aging studies of phosphorylated polysaccharides are few. For example, Shen et al [43] proposed that Tremella polysaccharide may be able to treat aging caused by oxidative stress. Zhang [44] found that polysaccharide from purple yam can realize anti-aging effect, too. Zhang et al [25] induced mice with D-galactose and found that excessive D-galactose may produce free radicals and lipid peroxides in mice, leading to obvious aging characteristics in mice six weeks later.

The indexes of thymus, liver, heart, kidney and spleen of mice were increased to varying degrees by adding *Trichosanthes* peel polysaccharide and phosphorylated *Trichosanthes* peel polysaccharide, which delayed the aging of immune organs. The results indicated that the polysaccharides of *Trichosanthes* peel and phosphorylated *Trichosanthes* peel had obvious anti-aging activity, and the phosphorylated *Trichosanthes* peel showed stronger anti-aging activity. This provides impetus to study the anti-aging activity of phosphorylated polysaccharides.

Immune Regulation Activity

Phosphorylated polysaccharide can activate the immune cells and improve the immune function of the body. Nagasawa et al [45] found that phosphorylated glucan promoted the mitosis of mouse spleen cells, enhanced the expression of CD86 and CD69 on the surface of B lymphocytes and dendritic cells, and promoted the expression of IL-10. The research results of Feng et al [46] showed that the phosphorylated *Radix Cyathulae officinalis* polysaccharides significantly increased the concentrations of IgG, IgA and IgM in immunosuppressed mice, promoted the proliferation of spleen cells, increased the thymus and spleen indexes, enhanced the levels of serum cytokines, increased the proportion of selective T cell subsets, and enhanced the cellular immunity and humoral immunity of the mice. Phosphorylated *Radix Cyathulae officinalis* polysaccharides also promote humoral and cellular immune responses by promoting dendritic cell maturation [26].

Antitumor Activity

The antitumor activity of phosphate may be related to the immune activation of phosphorylated polysaccharides. Chen et al [47] found that phosphorylation of (1 →3)-β-D-glucanexhibited relatively strong inhibition ratios against S-180 in vivo. Deng et al [14] found that polysaccharides from *Dictyophoraindusiata* did not inhibit the growth of tumor cells, but phosphorylated polysaccharides significantly inhibited the growth of MCF-7 and B16 tumor cells after the introduction of phosphate groups. He et al [48] found that both phosphorylation and sulfatation can significantly enhance antitumor activity, and compared with sulfatation, phosphorylation has a more obvious inhibitory effect on some specific tumor cells. The biological activities of polysaccharide derivatives are not only related to the degree of substitution and the site of substitution, but also related to the molecular weight, monosaccharide composition, solubility, chain conformation and so on.

Antiviral Activity

Phosphorylation of polysaccharides can improve their antiviral activity. Ming et al [49] found that the phosphorylation of *Codonopsispilosula* polysaccharide by STPP-STMP method could also reduce the liver injury of ducks infected with DHAV. This indicates that the phosphorylated polysaccharide of *Codonopsispilosula* had anti-duck hepatitis A virus type 1 (DHAV) activity. Later experiments showed that phosphorylated *Chrysanthemum indicum* polysaccharide inhibited the replication of DHAV genome, thus inhibiting DHAV infection in ducklings [50]. Wang et al [51] phosphorylated *Astragalus* polysaccharide (APS) to pAPS by STMP-STPP method, and sulfated APS to sAPS by chlorsulfonic acid-pyridine method. Later, observation by reverse transcription-polymerase chain reaction showed that pAPS had a stronger inhibitory effect on virus DHAV than sAPS and APS.

Anti-inflammatory Activity

China is the country with the largest export volume of glycosaminoglycan anti-inflammatory drugs, but polysaccharide drugs are characterized by large molecular weight and easy to be digested and degraded by oral administration, so further study is needed [52]. Ragab et al [17] found that phosphorylated rice straw hemicellulose (PRSHC) and phosphorylated palm frond hemicellulose (PPFHC) could significantly reduce the edema of foot plantar in rats. It showed significant anti-inflammatory activity. Therefore, PRSHC and PPFHC also have gastric protective effect. Li et al [53] found that the levels of IL-6 and TNF-α and related mRNA expressions were decreased in mice with chronic renal failure after gavage of phosphorylated *Pleurotusdjamor* polysaccharides. Through control experiments, it was found that the anti-inflammatory effect of phosphorylated *Pleurotus djamor* polysaccharides was superior to that of *Pleurotus djamor* polysaccharides, and the degree of alleviation of renal injury in the high-dose group was higher than that in the low-dose group.

Protect Liver Activity

Jing et al [54] found that the phosphorylated exopolysaccharide of *Lachnum*YM406 had a more significant effect on liver protection than exopolysaccharide of *Lachnum*YM406. Later studies have found that phosphorylated exopolysaccharide of *Lachnum*YM406 could reduce CCl_4-induced hepatocyte apoptosis by down-regulating the expression of Fas and Fas-L, decreasing the ratio of Bax/Bcl-2, accelerating PARP degradation and activating caspase 3/8. At the same time, the phosphorylated derivative can significantly alleviate liver fibrosis. Therefore, phosphorylated LEP-2a has a significant hepatic protective effect [55].

Other Activity

In recent years, new potential functions of phosphorylated polysaccharides have been discovered and studied continuously, such as anticoagulation, hemostasis, improving flame retardancy, improving drug delivery system, inhibiting corrosion, increasing swelling index, causing immunogenic response, and improving bone regeneration. Liu et al [12] found that phosphorylated *Sepia esculenta* Ink polysaccharide had stronger anticoagulant effect than ordinary *Sepia esculenta* Ink polysaccharide. Chitosan can covalently bind phosphate groups by means of mesulonic acid and phosphorus pentoxide, thus simulating the function of polyphosphate and showing hemostasis [56]. Shi et al [57] firstly synthesized chitosan-cobalt complex containing phosphorus and introduced it into polylactic acid matrix to improve flame retardant performance. Phosphorus pentoxide phosphorylates starch and hydroxypropyl starch and is loaded into the self-emulsifying drug release system, which can be developed and utilized as a new self-emulsifying drug delivery system [58]. Vimal Kumar et al [59] found that phosphorylated chitin can be used as a new environmental corrosion inhibitor. Rao et al [60] found that the swelling index of phosphorylated psyllium seed polysaccharide increased, which can be used as a gelling agent and suspension agent. Phosphorylated polysaccharides can also mimic the polysaccharides of *Clostridium difficile* in eliciting immunogen-like responses in mice, thus helping to design and select carbohydrate antigens as candidate vaccines [61]. Phosphorylated polysaccharide can also replace periosteum and improve bone regeneration, which can be applied in the treatment of bone regeneration [18].

Current situation and Prospect

Polysaccharides have wide sources, rich resources and broad development prospects. By means of biological activity determination and molecular modification, the derivatives with various structures and functions can be obtained. It lays a foundation for the study of polysaccharides, and provides a theoretical basis for the design, research and development of polysaccharides. It has been known that polysaccharides sulfation is one of the more mature modification methods. Its antiviral effect is remarkable. However, the acidizing modification is highly polluting and dangerous to operate. Recent studies have shown that phosphorylated polysaccharides also have antiviral properties and are more effective against certain viruses. Now more and more phosphorylated polysaccharides have been explored, their structure has been defined, their activity has been measured, and their applications have become more and more diverse, and they have been widely studied as a new therapeutic drug. It is believed that polysaccharide-phosphorylation will have a broader development prospect with appropriate methods.

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