

Review

# Progress in modifications and applications of fluorescent dye probe

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## Abstract

This review summarizes the labeling technology and applications of fluorescent dye probe in biology, especially the characteristics, modifications and applications of cyanine dyes. Based on the currently available modification methods of fluorescent dye probe, we discuss the studies of enhancing the water-solubility, improving the degree of biocompatibility and target-labeling, increasing the sensitivity and decreasing the toxicity of fluorescent dye. We also give a brief introduction on the modification method, that the fluorescent dye is directly introduced onto the cell surfaces by amine derivatives or azides to intensify the transferring information of aberrant cells. We suggest that fluorescent dye modified with chitosan oligosaccharide can obviously increase the degree of biocompatibility and target-labeling, and decrease the degree of toxicity.

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*Keywords:* Fluorescent dye probe; Cyanine dye; Chitosan oligosaccharide; Modification

## 1. Introduction

Cancer is one of the high-risk diseases threatening human life and health, and how to effectively restrain the occurrence and deterioration of cancer is becoming an urgent affair. With the advantage of low-price, safety and accuracy, biological molecular marker has attracted more and more attention widely in early-stage diagnosis in medical science. In 2005, National Cancer Institute of America (NCI) raised 9.8 million dollars to support the biological marker development laboratory on early detection research of network (EDRN), which engaged in developing biological marker technology related to cancer recognition. In the same year, Food and Drug Administration of America (FDA) also affirmed that the biological marker using fluorescent dye technology has a higher success rate, shorter test cycle, lower incidence of adverse reaction and fewer experimental patient numbers in clinical test. At present,

fluorescent marker technology has been successfully applied in the recognition of cancer and AIDS, and it is universally acknowledged as one of the convenient and effective methods for labeling tumor cells.

## 2. Fluorescence dye probe

Fluorescent marker technology is a process that fluorescent compound is combined with the labeled-object by physical or chemical action to form a complex. By detecting the fluorescence intensity, the message of the labeled-object is transferred qualitatively or quantitatively. With the development of modern medicine, molecular biology and various advanced fluorescence detection technologies and instruments including the application of flow cytometer (FCM), and laser scanning confocal microscope (LSCM), fluorescent marker technology has been appreciated extensively and gained swift development as a non-radioactive marker technology. The fluorescent marker technology can be used to determine varied kinds of substances, such as environmental pollutants, drugs, amino acids, chiral molecules, nucleotides [1], materials

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in-and-out side of cells. It also can be used to identify early-stage diagnosis of diseases, etc. and detect the process of life chemistry, such as enzymatic synthesis, immune response, protein biosynthesis [2].

As one of the widely used fluorescent labeling compounds (fluorescence dye probe), fluorescent dyes have many advantages, such as fast detection speed, good repetitiveness, low dosage, and non-radiation. Fluorescence dye probe is used to detect the structures of RNA and DNA, study the remedy of DNA damaged basic group, identify the status of amino group and the active area of protein molecule, detect protein in pmol scalar, distinguish nucleic acids with different conformation and the chemical reactive activities of related drugs. Fluoroimmunoassay by time-distinguished technique is used to analyze proteins, hormones, viral antigens and DNA hybridizations. The fluorescence spectroscopy induced by laser plays an important role in the identification of living cells, living body fluid, DNA base sequence and bacterial protomer, also in early diagnosis and treatment of malignant tumors [3].

### 2.1. Main fluorescent dyes applied in biological labeling

Fluorescent dyes applied in biological labeling are usually divided into two major classes in terms of the labeling modalities between fluorescent dye and the labeled cells. One is bound fluorescent probe bonding to labeled compound by active group, such as rhodamine dyes, fluoresceins, naphthalimides, stilbenes, acridine, and fluorenes. The other is embedded fluorescent probe inserting into DNA structure by affinity, such as thiazole orange (TO), oxazole yellow (YO), and their dimmers (TOTO and YOYO) of cyanine dyes. Comparing with former dyes, the latter class has many advantages, such as no fluorescence before inserting themselves into the nucleic acids structure and no background interference in the detection process. When it is inserted into a DNA molecule, especially the double-helix of DNA, the fluorescence intensity can be enhanced more than 1000 times, and more than 3000 times when inserted into RNA. This kind of dyes has higher affinity to tumor than to the normal cells [4], which will be widely used in the early-stage labeling of cancer cells. For biotechnology research, fluorescent dye probe has many advantages of high stability, high sensitivity and high selectivity. Considering the excellent performance of the cyanine dye mentioned above, we think the cyanine dye will play an important role in the fluorescent labeling analysis.

### 2.2. Characteristics of the cyanine dye

Compared with rhodamine and fluoresceins, cyanine dye has more advantages acting as a kind of fluorescent dye probe [5]. The most important characteristics of cyanine dye are the diversity and variability of its structure, from which many kinds of fluorescent labeling reagents

can be derivated. Multitudinous types of cyanine dyes are widely distributed from visible light to far infrared, in the absorption range of 350–1200 nm, with a great comparability and selectivity. Cyanine dye and its derivatives have large absorbance and can emit strong fluorescence after combining with bio-molecules. The complex can be detected by laser or fluorescence emitter. Multi-color or multi-parameter analysis can be conducted simultaneously on the active derivatives formed by dyes and bio-macromolecules, which is very advantageous to simplify operation, reduce cost, and improve the determining speed of different labeled components in one complicated mixed system. The strong fluorescence and relatively stable light of most cyanine dyes and their derivatives guarantee the fluorescence not to be quenched rapidly during the detection process with a fluorescence microscope. The dyes can also be used as simple and effective coupling reagents. Water-solubility and electric charge properties of the dyes can be adjusted by modifying their structures. Being low molecular weight (less than 1000), this kind of dyes has not steric hindrance to the reacting process and function of the labeled substance.

It is noteworthy that some of cyanine dyes are easy to be aggregated in aqueous solution, resulting in red shift in maximal absorption spectrum (a maximum 100 nm). The fluorescence intensity will decrease after the dye molecules aggregated themselves and obviously increase when connected with bio-macromolecules, meanwhile the stability will enhance in aqueous solution [6]. Incidentally, photobleaching under strong laser light is a common defect for organic fluorescence dyes.

Using cyanine dyes to label specific biological tissues has become a hot research area in the world. Mentioned labeling tumor cells with cyanine dyes modified by folic acid, Moon et al. found that due to their special affinity to folic acid, cancer cells showed fluorescence enhancement and prolonged retention time, which enhanced the targeting label effect to liver cancer cells [7]. Using cyanine dyes fluorescent probe modified by folic acid to study the optical imaging of aberrant tumor cells, Kennedy et al. found that tumor cells with folate receptor showed bright signals but normal cells hardly [8]. This method can distinguish tumor cells from normal tissues.

### 2.3. Application of the fluorescent dyes

Since fluorescent dyes are commonly lack of selectivity, fluorescent dyes fixed on biological activity carrier recognize aberrant cells selectively by using the selectivity characteristics among bioactivity compounds. For example, dyes combined with certain antibodies or some protein carriers are used to detect tumor, and in many anticancer drugs [9].

Due to their own recognition of certain kind of cells, some fluorescent dyes can combine with certain kinds of aberrant cells selectively to recognize them directly. Some

cyanine dyes with the characteristics of their own identification for certain kind of cells, such as merocyanine dyes and oxycyanine, can enter into cancer cells, and selectively label cancer cells or act as light poison to kill cancer cells. Simultaneously, this kind of cyanine dye can be used in solid tumor therapy as a kind of radiation sensitizer [10].

Furthermore, environmental factors can also be eliminated in the process of recognition of dissimilation cells by the fluorescence detection method. The high sensitivity of the method can even reach the molecular level. At room temperature, the latest research of fluorescence excited by laser in single molecule detection and single molecule spectrum provides a new method to study individual bio-macromolecule under physiological conditions, and to detect the spatio-temporal distribution of biological information changes [11].

### 3. Modification on fluorescent dyes

Design and modification fluorescent dyes can improve their application properties in the development of biological markers. So, transformation and modification of fluorescent dyes are much concerned.

#### 3.1. Design, synthesis and modification of fluorescent probe molecules

Modification of the molecular chain length of dyes is related to the formation condition of micelle, and the compatibility of functional dye molecules and cells is similar to gene transfer process. Studies on the modification of chitosan oligosaccharide by alkyl groups with different chain lengths showed that the closer the chain length to the micelle value, the better the hydrophilic-lipophilic of chitosan oligosaccharide. The carrier function reached to the best when 12-C alkyl was linked with a gene, which could be act as a basis for modifying dye molecules. Moreover, some modifications such as the improvement of optical sensitivity of fluorescent probe to DNA molecules or cells, the reduction of optical background, the elimination of cellular toxicity and the increase of water-solubility [12,13] have been achieved. There are some complementary relations among the diversified capabilities of dyes. For example, with the conjugated length of molecule prolonging, the stability of dyes usually decreases and the absorption wavelength increases; Maker activity improvement often results in the decrease of reaction specificity and the increase of hydrolytic rate. So, in order to design and exploit fluorescent probe with good properties, the relationship between dyes' properties and molecular structures should be emphatically studied. Some factors have important effects on biological application, such as remarkably fluorescent properties, photo-stability, solubility, sensitivity at low pH solution, possibility of preparing different types of active derivatives, low internal toxicity and good bioactivity.

#### 3.2. Synthesis methods of cyanine dye

Hot topics in synthesis methods are optimizing synthetic methods, shortening synthetic route, improving yield, exploring effective separation methods, and attaining the methods with high purity fluorescent probe molecule [14–16]. Traditionally, 1-alkyl-4-methylquinolinium salt and 2-methylthio-3-methylbenzothiazolium salt are used to synthesize TO derivatives [17], in which expensive iodomethane is used as a methylation agent. Moreover, this reaction also produces a byproduct  $\text{CH}_3\text{SH}$ , which is a noxious gas and may result in serious environmental pollution.

By melting 6-substituted-2-aminobenzothiazoles and 1-alkyl-4-methylquinolinium in vacuum condition, Deligeorgiev et al. [18] synthesized 6-substituted TO derivatives. This method synthesized not only symmetrical but also asymmetric monomethine cyanine derivatives. The preparation procedure is simple and quite applicable to synthesize 6-substituted TO derivatives because the starting materials are commercially available or easily synthesized. Cyanine dyes also was synthesized from 4-chloroquinolinium salt and 4-methylquinolinium salt in the presence of triethylamine [19]. Cyanine dyes have been synthesized by the reaction of quaternary ammonium salts of a heterocyclic 2- or 4-methyl compound; and products can be gained by melting or boiling N-heterocyclic 2- or 4-sulfobetainic compounds in the absence of a basic compound. Both the two methods emitted toxic gas  $\text{SO}_2$  [20]. By condensing 3-alkyl-2-methylbenzothiazolium salt and 1-( $\omega$ -bromoalkyl)-4-quinolinium salt in the presence of triethylamine, TO derivatives could also be synthesized [21,22]. In recent years, a method of preparing indole cyanine dyes by solvent-free microwave irradiation has been paid much attention because of its safe process, simple device, high speed, easy purification of products, and high yield [23].

The process of separating and purifying cyanine dyes in solid-phase synthesis often presents easier operations and more performances than that in the liquid-phase reaction [24,25].

In 2007, we proposed a new solid-phase synthetic method to synthesize cyanine dye TO and derivatives, which realized the traceless cleavage based on Merrifield resin method [26]. Our method simplifies the process of synthesis and posttreatment, and is easy to modify different activity sites and bridged chain of TO and derivatives. The synthetic route is shown in Fig. 1.

#### 3.3. Targeting labeling between fluorescent probe and cancer cells

Dyes molecules can be modified by appropriate ligands to realize targeting label on aberrant cells through interaction between ligand and receptor. Ye et al. [27] synthesized cyanine dyes with multi-glucosamine and gained a kind of polydentate compound with near-infrared carbocyanine dye as a core. Polydentate ligand probe is more stable than the precursor dye in physiological conditions, and has bet-

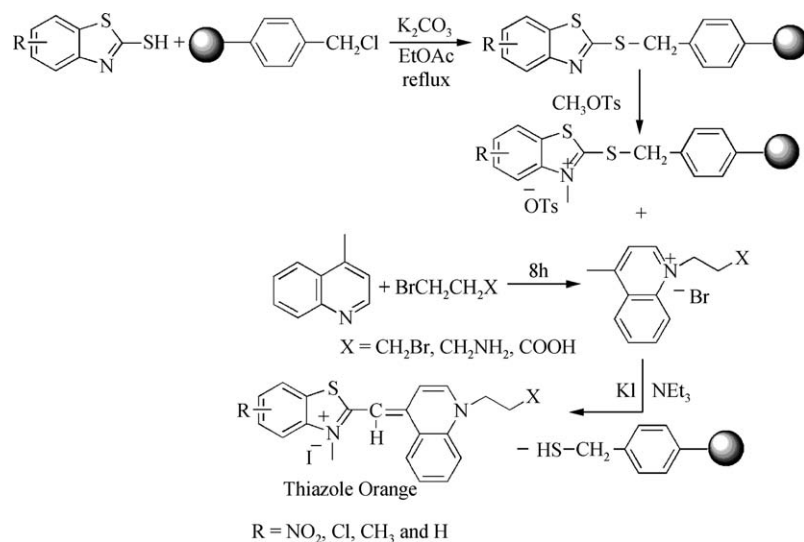


Fig. 1. Synthetic route for TO and derivatives.

ter targeting label function for tumor detection. Experiments on mice carrying a “CA20948” tumor showed that all the glucosamine conjugates cyanine dyes had ideal target function for labeling tumor. Histocompatibility of cyanine dyes was enhanced by the mechanism of synergetic effect [27].

Quantitative studies on endothelial tumor cells labeled by cyanine dyes showed that tumors connected with endothelial cells in circulation system are very important to tumor cell diffusion. Linking a fluorescent cyanine dye molecule to endothelial tumor cells quantitatively, researchers could observe cells images with high fluorescence marker and with no obvious cytotoxicity [7]. Labeled tumor cells can be used to evaluate endothelial cell layers and detect fluorescent plate. Results of a luminoscopic test showed that there was no tumor metastasis between tumor cells and linked endothelial cells. The fluorescence signal remained detectable after three days, and with obvious cyto-toxicity. These results indicated the security of using these cells and cyanine dyes.

### 3.4. Improvements of strength and sensitivity fluorescent dyes

The key research points on biological fluorescent probes are improving water-solubility of dyes, increasing sensitivity, enhancing optical stability and obtaining excellent biological tissue marker target. However, there are some insurmountable defects in biomedical research for traditional fluorescent analysis. For example, there always exists photo-bleaching when traditional chromophore rhodamine 6G or other organic dyes molecules are used in fluorescent analysis method, resulting in the instability of fluorescent signals. Emission spectral peak of the organic fluorescent dyes is often located closely to the excitation spectral peak with an asymmetric shape and a serious tail of peak. Fluorescent overlapping exists among different emission peaks.

So it is difficult to excite several kinds of fluorescent dyes (usually no more than three kinds) by the same excitation light sources simultaneously [28]. Nevertheless, due to its excellent spectral properties and photochemical stability, semiconductor nanoparticles can be used as a probe to overcome the various disadvantages of organic dyes. It was reported that chitosan was used to modify fluorescent quantum dots and improve its water-solubility so as to apply them in bioluminescence labeling [29].

In order to enhance fluorescein sensitivity within a certain concentration range, Zhou and his colleagues [30] added nanometer silver particles into fluorescein solution. Their experimental results showed that nano-silver particles transferred energy to the fluorescein luminescent center, thus achieved the fluorescence enhancement by producing strong localized fields [30]. This method can effectively reduce the quenching phenomena of organic dyes.

Cyanine dye molecules attached to the surface of an engineered virus showed a tissue-specific distribution pattern. Energy can be transferred from the virus to adducted dye molecules, resulting in highly fluorescent viral nanoparticles. With this enhanced fluorescence DNA–DNA hybridization could be detected. Compared with the most commonly used detection methods in microarray-based genotyping assays, these viral nanoparticles markedly increased assay sensitivity, and thus are applicable for existing DNA microarray protocols [31–33].

In the recent years, researchers designed and synthesized polymer fluorescent probes in the scale of small molecule monomers, and obtained polymer supported fluorescent probes by copolymerizing with other monomers. Experimental results showed that polymers obtained by different polymer monomers have different selectivities to different objects, which provides solid basis to design practical polymeric fluorescent probes in the future.

#### 4. Fluorescent dyes bound onto cells surfaces by amine or azide derivatives

Fluorescent DNA hybridization probes prepared from DNA and active dyes modified by amine or azide derivatives can greatly improve the sensitivity of cell markers, which is difficult to achieve in traditional ways [34].

Bertozzi et al. presented a new method of introducing contrast reagents onto cell surfaces [35,36], and *N*-leyulinolmannosamine (Menlev) or *N*-acetylacetone alcohol were incorporated into cell membrane through metabolic processes of these reagents with the glycoprotein on the cell's surface (Fig. 2).

It was reported that introducing an azide group to sialic acid on the surface of aberrant cells led to a Staudinger-Ligation reaction between fluorescein and azide on cell surface. Thus fluorescent dyes could be incorporated into cell membrane and express the information of aberrant cells with strong directionality (Fig. 3). This reaction is of high selectivity. Dyes with an active group can be oriented to cell's surface containing azide. In this process no interference occurred from the existing functional groups in cells or tissues. Thus the target orientation of fluorescent probe could be enhanced.

Enhancing the compatibility between fluorescence probe and human tissue, improving the target orientation of fluorescence probe, increasing the sensitivity and decreasing the toxicity are major concerns of further development for fluorescent probes. Recently, fluorescent dyes modified by bioactive polysaccharides have got much attention. A hot topic in the recent study is that fluorescent dyes are connected with chitosan to enhance histo-compatibility and improve the target orientation of fluorescent probe, as well as increase sensitivity and decrease toxicity.

#### 5. Fluorescent dyes modified by bioactive polysaccharides

##### 5.1. Structures and characteristics of polysaccharides

Polysaccharides widely exist in organism and can be regarded as one kind of important information molecular receptor. They not only provide energy, participate in structural organization in organism, but also play roles in cell reorganization, cell growth, cell differentiation, cell metabolism, embryonic development, cell canceration, immune response and other various life activities. Therefore, studies on polysaccharides have attracted biologists, chemists and pharmacologists' interest, and some experts

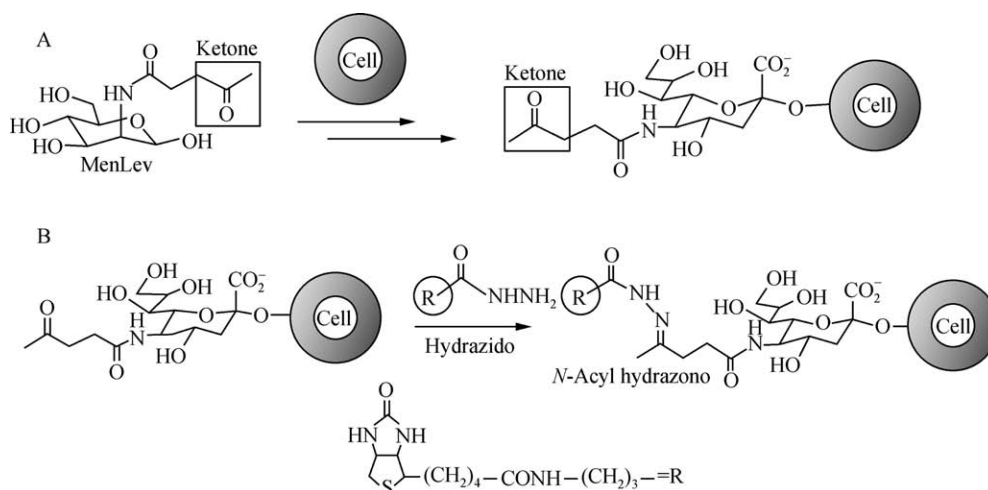


Fig. 2. Monley incorporated onto cell membrane.

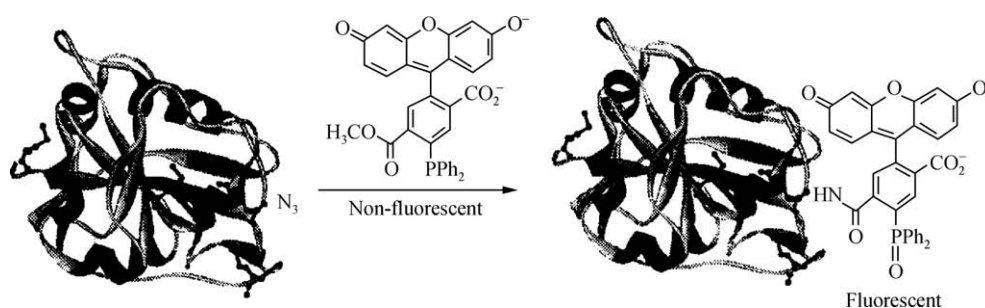


Fig. 3. Staudinger-Ligation reaction between azide and fluorescein.

foretold that “the 21st Century will be the era of polysaccharides” [37].

Biological activity of polysaccharides is affected by the modification on their spatial structures, and the changes of their molecular weight, types, number and positions of substituting groups. So, polysaccharide molecules have been modified properly into different derivatives to improve biological activity. Thus, the modification of polysaccharide molecules has become a key way to enhance biological efficiency of polysaccharides. The activity of polysaccharide is closely related to certain chemical groups in its molecule, which are added or eliminated by artificial chemical reaction for the purpose of increasing polysaccharide activity and probing their relationships between structure and effectivity.

### 5.2. Fluorescent dyes modified by bioactive polysaccharides

As a drug delivery system, polysaccharide could be changed to a polymer carrier through cross-linking. Besides its slow and controllable release, this carrier system also has high compatibility, biodegradability, antigenicity, anti-inflammation, antiviral, infection and other favourite biological properties. The lead compound modified by polysaccharide not only prolongs the action time of drug and enhances the effect, but also releases drugs on target and decreases toxic side effect [38,39].

The anti-tumor function of bioactive polysaccharide is realized by a series of mechanisms, such as regulation and improvement of organism's immunity, anti-oxidation and anti-mutation on free radicals, radiation resistance, inducement of cancer cell differentiation and apoptosis, and elimination of cancer cells. Thus fluorescent probe modified by polysaccharide can provide a new way for tumor diagnosis and treatment. Main representatives of bioactive polysaccharides in this field are sodium alginate and chitosan.

Sodium alginate is a furfural acid mainly composed of polysaccharide, which is constituted by L-gulonate and D-mannuronate in linear polymers. Due to its special colloidal properties and other characteristics, such as thickening ability, stability, suspensibility, film forming properties, and sodium alginate is widely used in daily chemical, dyeing, printing, and pharmaceutical manufacture industry. Furthermore, sodium alginate has been widely used in the study of gene and drug delivery by a kind of carrier because it contains hydroxy, carboxyl and aldehyde groups which participate in the reaction.

Chitosan is a cationic polysaccharide, which is mainly composed of repeating units of  $\beta$ -(1–4)-2-amino-2-deoxy-D-glucopyranose, with a small amount of N-acetyl-D-glucosamine. This basic polysaccharide of glycosaminoglycans type can be easily combined with cell wall acid receptor residues on cell membrane, so it can be directly combined with cell membrane. Chitosan is easily modified because of its excellent chemical activities. Moreover, chitosan can modify selectively by appropriate biological molecules for a spe-

cial purpose. For example, ligand positioning receptor and DNA probe modified by chitosan can be used to detect the target and molecules. As a nonviral vector, galactosylated chitosan showed good targeting effect when used in gene therapy for hepatocellular carcinoma [40].

Kim et al. [41] used fluorescence dyes modified by galactosylated chitosanthiocyanate to get targeted image of hepatocytes. After injecting dyes to mice via the tail vein, optical images were acquired through a  $\gamma$ -camera equipped with pinhole collimators. After 10, 60, 120 min, optical signals were found to be mainly located in hepatocytes. Folic acid ligands were observed to first combine with receptors on specific targeted cell surface, and then enriched in a certain region of the target cells by the endocytosis process. So, chitosan acts as a bridge between ligands and dyes, thus enhancing the cellular compatibility with dyes and reducing toxicity. Chitosan combined with many dye molecules can enhance fluorescence, which is also helpful to increase the sensitivity and decrease the consumption of probe molecules [42].

## 6. Conclusions

In this paper, we briefly review the characteristics, applications and modifying methods of fluorescent probe molecules used in biological labeling. For developing bio-fluorescent probes, the improvements of their sensitivity, stability and enhancement of their recognition ability to aberrant cells are key issues. Some bioactive polysaccharides show remarkable anti-tumor activity and other biological activities, and also are nontoxic. Fluorescent dyes modified by bioactive polysaccharides can increase biocompatible and targeting effect, decrease toxicity, and improve sensitivity.

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