光散射技术及其在生化药物分析中的应用

Light Scattering Techniques and Their Applications in Biochemical and Pharmaceutical Analysis

黄承志 谭克俊 李原芳 编

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黄承志　谭克俊　李原芳　编

责任编辑：杨光明　杨浩宇

特约编辑：张迎雪

封面设计：周　娟　钟　琛

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前　言

散射光产生于光子与微小粒子的相互作用。当光子作用于任何不均匀介质时，都有散射光产生。结合激光技术，散射光已在胶体化学、高分子化学等粒子大小表征和分子量测定方面得到了应用，但在吸收和发射(荧光)光谱分析中，散射光却是严重的干扰因素。1993年，美国学者Pasternack及其合作者发现使用普通荧光光度计获得的光散射信号可应用于生物色素聚集体研究。在此基础上，我们把普通荧光光度计测定的光散射信号与物质的量联系起来，建立了以散射发光为检测信号的光谱分析法。近十年来，基于散射光信号的光谱分析法已为分析工作者广泛应用于无机离子、蛋白质和核酸、药物的测定中，发展十分迅速，并随着纳米科学的发展取得了新成就。

作为近年来工作的总结，我们选编了本论文集。内容主要涉及到我攻读博士学位期间完成的工作和我在西南大学所指导的研究生的论文。在此，感谢我的恩师北京大学童沈阳教授及其课题组的李克安教授、刘锋教授、赵凤林教授和李娜教授等的指导和帮助，感谢我的研究生们近年来为光散射分析所付出的努力，并希望他们在今后的工作中取得优异成绩。

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黄承志

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Contents

Chapter 1

Developing Process of Light Scattering Technique Analysis

1.1 Resonance Light Scattering Technique Used for Biochemical and Pharmaceutical Analysis

Abstract: By coupling and scanning simultaneously excitation and the emission monochromators of a common spectrofluorometer, enhanced resonance light scattering (RLS) signals could be obtained. The enhanced RLS signals could be used for designating bio-assemblies, aggregation species, and analytical purposes. Herein, we review the reports since the year of 2000 concerning the biochemical and pharmaceutical analysis with the RLS measurements, and discuss the possible developments of this technique.

Keywords: Resonance light scattering (RLS) technique; Biomolecule; Biochemical analysis; Pharmaceutical analysis

1.1.1 Introduction

Light scattering exists starting from our daily life to universal space. Beautiful unforgettable views of rainbows, sunrises and sunsets originate from scattered rays of photons interacting with particles such as dust, cloud and smog in the medium like air[1]. Scattered lights can emit in all directions except that of the incident light beams propagated, and are strongly related to the inhomogeneity of the medium. Observations of scattered lights are very common since all media except vacuum are relatively inhomogenous[2,3]. Depending on the size of the photon-interacted particles *(d)* and the wavelength of incident light beams (λ_0), light scattering can be classified into Mie ($d>>\lambda_0$), Tyndall ($d \approx \lambda_0$), and Rayleigh scattering ($d \leq 0.05\lambda_0$) [2]. In terms of the wavelength, difference of the incident and the scattered beams, light scattering can also be divided into elastic scattering, inelastic scattering and quasi-elastic scattering[3,4]. Rayleigh scattering is elastic scattering, while Raman scattering and Brillouin scattering belong to inelastic scattering. Quasi-elastic scattering results from the Brownian movement of the scatterers, and can be named as dynamic light scattering[3,4].

These light scattering phenomena, if coupled with laser technique, have been extensively applied to polymer, colloidal and pharmaceutical sciences including measuring the size and distributions of polymer particles[5,6], col-

loids[6~8], drug powders[9] and selfassemblies of biopolymers [10]. In analytical chemistry, light scattering detectors have been developed in chromatographic determinations based on measuring the signals of multiple light scattering[11,12] and multi-angle light scattering (MALS) [13,14]. Evaporative light scattering detections (ELSD) have been developed for aerosol converted from the effluent of the separation in capillary electrophoresis[15] and HPLC[16], acting as complementary methods in terms of universality ensuring that LC–MS–ELSD run can provide sufficient MS and retention time data for identification of known present. ELSD also supplies quantification with a great degree of accuracy than UV detection for the analysis of combinatorial libraries[17].

On the contrary, light scattering is one of key interference sources in spectrophotometry and spectrofluorometry. Techniques such as low temperature and magnetic field-resolved techniques have been proposed to reduce their effects[18], and mathematical methods have been put out for separating the scattering and absorptions in the geometrical arrangement of interacting chromophores in order to avoid misleading investigations of the experimental spectroscopic parameters[19,20]. However, the establishment of resonance light scattering (RLS) technique, which measures light scattering signals on a common spectrofluorometer, has greatly changed the situation of the light scattering in spectral measurements [21]. Due to the simplicity of the technique, its applications have been developed greatly in recent years, and there have been several mini-views standing at different positions displayed[22–25] based on the reports before the year of 2000. Considering that analytical methods are basically established on the enhanced RLS signals resulting from the aggregations or assembly of chromophores on biomolecule template, starting from that point herein we make a mini-view with the reports since the year of 2000.

1.1.2 Basic theory

For particles with their size less than 20-fold of the wavelength of incident beams (20 $d \leq \lambda_0$), their scattered light could be described by Rayleigh theory, which assumes that all of the electrons in a particle *oscillate* with the same phase and frequency as the incident electromagnetic wave, and this collective oscillation results in a large oscillating electric dipole moment that produces scattered light. The produced light scattering intensity could be expressed by Rayleigh equation [21,22,26]:

$$I = I_0 \frac{8000(2.303)^2 \pi V n^2 c}{3\lambda_0^4 N_A} \left\{ \frac{\varepsilon^2(\lambda_0)}{4\lambda_0^2} + \left[\frac{1}{\pi} \int_0^\infty \frac{\varepsilon(\lambda) d\lambda}{\lambda_0^2 - \lambda^2} \right]^2 \right\} = Kc \quad (1)$$

where I is the intensity of the light scattering, I_0 the intensity of incident light beam, λ_0 and λ the wavelength of the incident and scattered light beams, respectively, c the concentration of the scatterers, and N_A the Avogadro constant. The terms in brackets indicate the real and imaginary parts of the refractive index, respectively.

Eq. (1) shows that in the non-absorption medium, the light scattering is mainly dependent on the real part of the refractive index *(n);* while if the excitation wavelength is near to the absorption bands, both the real and imaginary parts of the index make the contributions to the light scattering. If the absorption is strong, the contribution of the imaginary part is very significant since the fluctuation of the complicate index is very steep, strong enhanced Rayleigh scattering signals could be expected[21]. The enhanced light scattering could reach the maximum when the real part of the refractive index is zero and the imaginary part relative to the medium is 1.414[27]. These enhanced light scattering signals can be measured by using a common spectrofluorometer through simultaneously scanning the excitation and emission monochromators of a common spectrofluorometer with the wavelengths of

the two monochromators being equal (namely $\Delta\lambda = 0$)[21].

It should be noted that electrons in different parts of the particle oscillate with different phase when the particle is comparable to or larger than the wavelength of incident beams. This leads to interference of the light scattered by electrons in different parts of the particle, and the magnitude and angular distribution of the scattered light intensity then deviates from that of an oscillating electric dipole [1]. However, according to theoretical calculation, Eq. (1) could be extended to the Mie scattering at some proximity, for example, to the light scattering of 40 nm gold particles excited by 500 nm incident beams [27]. In addition, the RLS measurements by using a common spectrofluorometer are generally operated with comparatively large slit-width for excitation and emission ($\geq$ 5 nm). It has proved that $\Delta\lambda$ is not always zero if maximal RLS signals are available when large slit-widths ($\geq$ 1 0 nm) are employed[28]. Without optical correction, the stray lights in the optical system of the spectrofluorometer obviously exist. Thus, RLS signals, obtained by using a common spectrofluorometer, involve in not only pure Rayleigh scattering, but also other light scattering signals including Mie, Tyndall, and Brillouin light scattering. Even though, as following will demonstrate, RLS is a valuable technique for detecting and characterizing self-assemblies and extended aggregates of chromophores since the assemblies or aggregations lead to formation of large fractal structures exhibiting strong RLS signals [29].

1.1.3 RLS measurements to assign assemblies

1.1.3.1 Self-assemblies of chromophores

Self-assemblies of porphyrins stand out of supramolecular chemistry since monomers of porphyrins can spontaneously self-assemble into dimers or higher aggregates through π-π stacking interactions and σ-π attractions, supplying the possibilities to mimic complicate physicochemical processes[30], and to build blocks for the rational design of self-assembled supramolecular structures in nanoelectronics applications, and the synthesis of materials capable of exhibiting specific properties and functions[31].

Water-soluble porphyrins bearing charged groups could be fostered to aggregate by simply screening the repulsive interactions between side-groups with the same charge, thus aggregation of porphyrins in variety of media have been investigated by spectroscopic techniques including RLS[32]. The dimers or higher aggregates of anion porphyrins such as tetraphenyl sulfonate porphyrins ($TPPS_4$), and tetraphenyl carboxylic porphyrins (TCPP) formed in aqueous media generally display enhanced RLS signals around the absorption bands of the J- and H-aggregate species since the absorptions of the J- and H-aggregate species are strong, and the contribution of the imaginary part of the complicate index of the aggregates is very significant[22,33]. The amount of RLS signals increases with a "good" electronic coupling among the porphyrin chromophores, whereas corresponding signals were absent in monomers [34]. Furthermore, RLS measurements have shown that H^+ and Na^+ in aqueous medium have displayed different driving ability to the final mesoscopic structure formed from the J-aggregation of $TPPS_4$ disclosing that the aggregation number ranges between 6 and 32, and even if a aggregation number range of $10^5 \sim 10^6$ in the case of the large clusters[35]. Similarly, nanoscale self-assembled complexes of chlorophyll in organic solvents could also be investigated by RLS measurements, and it was found that the formation of spherical-shape aggregates in which the phyil-chain is segregated in the inner part could foster the pigments so as to expose the macrocylic heads toward the bulk water solvent[36].

Since the π-π and σ-π interactions of porphyrins depending on the molecular structure mainly involve the substitute at the periphery, a variety of cationic and anonic porphyrins have been synthesized in order to clarify the

factors influencing self-aggregation tendency. By means of absorption and RLS spectroscopy, Pasternack and co-workers proved that the aggregation of water-soluble porphyrins is dependent on the peripheral mesosubstituents[37]. Thus, it is easily understand, as derived from depolarized RLS measurements, that the half neutralization of carboxylic acid side chain of Protoporphyrin IX, the iron-free form of hemin, is responsible for the occurrence of a network of intermolecular hydrogen bonds contributing to a better stabilization of a supramolecular assembly, which display a prolate shape with an average of 200～500 nm and a medium height of 60 nm by evaporating solutions of the Protoporphyrin IX dimers[31].

1.1.3.2 Assembly of chromophores on charged polymeric template

By coupling the absorption, fluorescence, circular dichroism (CD) spectra and RLS measurements, a different approach could be made based on the electrostatic interaction between charged porphyrins and oppositely charged polymeric matrices including surfactants, nucleic acids, proteins [38], and hindered oppositely charged porphryins, where they act as template supports [39]. Pigment-surfactant aggregate of chlorophyll could be designated with induced RLS spectra in the micellar solution, in which the positive charge of the surfactant interacts with the C-13[2] ketoester group of monomeric porphyrin and form a delocalized exciton state[40].

Unlike free base porphyrin, such as $TPPS_4$, TCPP, and Protoporphyrin IX, metalloporphyrins display different aggregation features induced by ionic and nonionic surfactants. Metalloporphyrin including $FeTPPS_4$ and $ZnTPPS_4$, either monomers or aggregates, could bind to micelles, forming non-micellar metalloporphyrin/surfactant aggregates, and the binding ratio and constant of metalloporphyrin/$TPPS_4$, which could be made with UV, fluorescence and RLS measurements, are varied depending on the metal ions since the metalloporphyrins are incorporated into the micelles near the terminal part of their hydrocarbon chains[41,42]. Copper(II) porphyrins, if differing in peripheral substituents when interacting to DNA template, are either intercalated, bound externally or form extended electronically coupled arrays, and the extended electronically coupled arrays have characteristic RLS spectra corresponding to their absorption bands[43]. Similarly, induced RLS signals concerning the binding and self-organization cation porphyrin onto the long-range assemblies on poly(dA-dT)$_2$ or poly(dG-dC)$_2$ indicate that exciton coupling occurs between adjacent porphyrins moieties, and higher hydrophobicity of porphyrin is manifested by more extensive self-organization[44].

Depending on the substituted β-cyclodextrins (β-CDs), 2:1 supramolecular inclusion of the β-CDs with tetrakis(4-methoxyphenyl)porphyrin follows obvious RLS enhancement of the porphyrin, and is related to the size of the alkylated substituents interactions[45]. On the contrary, trans-bis (*N*-methylpyridnium-4-yl) diphenylporphyrin forms extended and organized assemblies on DNA template, but the addition of β-CD to these arrays leads to their disassembly as evidenced by the changes in extinction, CD and RLS spectra since β-CD attack the interior of the porphyrin assemblies as well as the ends [46]. Similar to the inclusion of β-CDs with porphyrins, induced RLS signals have been employed to designate the 1:1 inclusion complex of procaine hydrochloride into β-CD with the formation constant at 1.2×10^2 mol·L^{-1}[47,48].

RLS measurements show that the aggregation of $TPPS_4$ induced by cationic cyanines with different substituents results from the hexyl-chain, indicating that it is the chain length of the alkyl group attached to the benzoxazole in the cyanine dyes for stabilizing the structure of the aggregate[49] that induced the aggregation of $TPPS_4$. The porphyrin's aggregation kinetics display an induction period unlike that of the cyanine dye[50], while the assemblies of carbocyanine dyes display a dependence on the substituents differing complex supramolecular structures of nanometer-to-micrometer size, that could be detected by coupling RLS and cryo-TEM [51]. In addi-

tion, G-quadruplex DNA could selectively bind to perylene diimide depending on the pH-dependent aggregation of chromophores[52,53]. The pH-dependent assemblies of chromophores can be used as pH sensors, and these supramolecular aggregates can also be employed as sensors for DNA [54].

1.1.4 Factors deciding enhanced RLS intensity

As shown in Section 3, RLS technique is powerful to designate the assemblies or aggregations, and it is generally combined with the absorption, fluorescence, and CD measurements in order to assign spectral features. Besides that, we can establish analytical methods through Eq. (1) by measuring enhanced RLS intensity in the aggregation and assembly process as stated above. Tables 1～3 list the RLS methods of biochemical and pharmaceutical analysis since the year 2000[55～136]. However, here we only stress on common features of the RLS signals extracted from these numerous reports, and will not fall in to the diversity of the interaction system even if these methods are generally established based on the interaction of the analytes with chromophores holding with different molecular structures, since a general summary of these interactions is beneficial to developing new analytical methods regarding the choice and synthesis of new chromophores. Based on extensive studies from the molecular recognition, assemblies and aggregations of chromophores induced by biomolecules, we suppose that enhanced RLS signals at least depend on following factors on the basis of the formation of delocalized exciton state of the assemblies.

1.1.4.1 Structural compatibility of the interacting components

The factors of structural compatibility include the interacting components' size, symmetry, charges and the solubility in appropriate solvents[36,37,55]. The interaction-induced enhanced RLS signals were thought to result from the size difference and the charge-coupled transfer of the scatterers before and after the interaction due to the electrostatic or hydrophobic interactions[66～68]. That is, the enhanced RLS intensities are related to the charge properties of chromophores, the charge-coupled degree of the formed species related to the intramolecular electronic interaction, and the size of the formed species. Thus, the strong electrostatic interaction between the two interacting components inducing high electronic delocation, and the formation of large particles are compulsory to display characteristic RLS spectra[33,63]. For the interactions between chromophores with proteins[66～97] or nucleic acids[103～117,122～128], the general rule is that positively charged chromophores interact with nucleic acids while negatively chromophores with proteins.

Table 1 Determinations of saccharides and medicines with enhanced RLS signals

Saccharide, medicines	pH	λ^a (nm)	Linear range ($\mu g \cdot mL^{-1}$)	LOD (3σ, $ng \cdot mL^{-1}$)	Reference
Heparin-Victoria blue 4R (VB-4R)[b]	5.0～7.6	523	0～0.4	3.35	[55]
Heparin-Victoria blue B (VBB)	5.0～6.4	534	0～0.4	6.62	[55]
Heparin-Night blue 4R (NB)	5.0～6.4	554	0～0.4	6.69	[55]
Heparin-Crystal violet (CV)	6.0～11.2	492	0～1.2	2.9	[56]
Heparin- Methylene blue (MB)	5.0～6.0	346	0～0.6	9.0	[57]
Heparin-Azure B	4.0～7.0	338	0～1.2	14.5	[57]
Heparin-Toluidine blue (TB)	4.0～7.0	329	0～0.8	26.0	[57]
Heparin-Brillint cresol blue (BCB)	6.0～6.5	348, 545	0～5.0	/	[58]

Continued

Saccharide, medicines	pH	λ^a (nm)	Linear range ($\mu g \cdot mL^{-1}$)	LOD (3σ, $ng \cdot mL^{-1}$)	Reference
Chondroitin 4-sulfate (CS)- Brillint cresol blue (BCB)	6.0～6.5	360, 544	0～5.0	/	[58]
Glycogen-Concanavalin A	7.4	400	0.32～24.0	/	[59]
Glycogen-Aqueous solution	2.5～11.5	350	0.8～4000	/	[60]
Thiamine (VB1)-Sodium dodecylbenzene sulonate	3.29	375, TIR-RLS	0.12～0.8	0.12	[61]
Chlortetracycline (CTC)-Eu-Trioctyl phosphine oxide (TOPO)	7.54～8.14	340, TIR-RLS	$0.98 \sim 20.0 \times 10^{-7}$M	9.8×10^{-9}M	[62]
Berberine-Acidic xanthene (Eosine Y, Erythrosine, Ethyl eosin, Phloxin, Rose bengal)	4～5	576,578,572,384,464	$0 \sim 5.0 \times 10^{-6}$M	14.3,30.8	[63]
Raloxifene-Evans blue (EB)	1.8	470,400	0～8.3	18.9	[64]
Vitamin B1(VB1)-Methyl orange	2.3～3.0	588	0～0.4	7.2	[65]

[a], Maximal RLS wavelength; [b], analytes were listed before the dash and after that the chromophores were presented.

1.1.4.2 Environmental conditions

If the interaction results from electrostatic attraction between biomolecules and chromophores, it is necessary that they should have opposite charges. Thus, these interactions originating from the negative charges of phosphate in the molecules of nucleic acids and the positive charges of proteins should be controlled under appropriate acidity and ionic strength, since the change of acidity and ionic strength of the medium would change the charges of both interacting components and the conformation of the biological molecules[73～78].

Different RLS signals could be obtained when different proteins bind to same chromophores since the number of $-NH_3^+$ of different proteins are greatly different[23,24]. For example, the number of the alkali amino acids residue in protamine sulfate is much more than that in bovine serum albumin (BSA), and the obtained RLS signals concerning the interaction of negatively chromophores with protamine sulfate are much stronger than that with bovine serum albumin [24]. On the contrary, the isoelectric point of pepsin is 1.0, and its interaction with negatively charged dyes seems impossible in common acidic medium [93]. For the interaction of proteins with chromophores, suitable pH for the interaction is generally lower than the isoelectric point of proteins[68]; while the appropriate pH for the interaction between nucleic acids with chromophores mainly depends on the protonization constant of chromophores[108,109]. As to these interactions of proteins with chromophores mainly through electrostatic force, RLS intensity is in proportional to molecular weight of proteins in Dalton[93]. That finding could be used for molecular weight measurements.

Besides acidity and ionic strength of the medium, temperature has effect also. With the increase of temperature, Brownian movements of molecules are encouraged, that induces more dynamic light scattering signals since RLS signals were measured generally with large slit-width (≥ 5 nm), and the direct outcome is the instability of the RLS signals[28].

Table 2 Determinations of bovine serum albumin on its enhanced RLS signals on chromophores

Chromophores	pH	RLS peak (nm)	Linear range ($\mu g \cdot mL^{-1}$)	LOD (3σ, $ng \cdot mL^{-1}$)	Reference
Anion porphyrins					
$\alpha, \beta, \gamma, \delta$-tetrakis(4-sulfophenyl)porphine ($TPPS_4$)	1.86	490.2	0.15~0.8	18	[66]
Triphenylmethane					
Fuchsine Acid (FSA)	4.10	277	0~3.8	0.47	[67]

Continued

Chromophores	pH	RLS peak (nm)	Linear range ($\mu g \cdot mL^{-1}$)	LOD (3σ, $ng \cdot mL^{-1}$)	Reference
Fast Green FCF(FCF)	4.10	279	0.02~2.0	1.86	[68]
Xanthene dyes					
Pyrogallol red (PR)	3.6~4.2	347	0.25~13.0	51.0	[69]
Azo dyes					
4-Azochromotoropic acid phenylfluorone (ACAP)	0.5~1.8	337	0.2~4.0	68.0	[70]
Dibromochloroarsenazo (DBC-Arsenazo)	/	/	2.5~6.0	88.1	[71]
Dibromomethyl-Arsenazo-Al(III)	5.6~7.2	410	2.5~50	135.0	[72]
Dibromomethylchlorophosphonazo (DBM-CPA)	3.78~4.35	411.6	0.065~40.05	30	[73]
Amaranth	3.87	364	0.5~5.0	/	[74]
Monoazo dyes (Chromazol KS, Acid chrome dark blue, Chrome blue SE, Acid chrome blue K, Chlorophosphonazo I, Arsennazo I, Chromotrope ZR)	3.8,3.2~4.2, 3.4~4.2	338, 314, 330, 336, 338	1.0~7.0	10	[75]
Th(IV)-Bisazo dyes of chromotropic acids:					[76]
arsenazo III (AA EII),	Acidic medium	470	0~2.0	13.6	[76]
arsenazo M (AA M),	Acidic medium	470	0~2.8	22.1	
chlorophosphonazo HI (CPA III),	Acidic medium	470	0~1.6	10.7	
chlorosulphonphenol S (CSP S)	Acidic medium	470	0~0.28	6.3	
Orange G	0.6~2.0	548	0.5~5.0	2.6	[77]
3-(4-Sulphophenylazo)-4,5-dihydroxy-2,7-naphthalene disulfonic acid (SPADNS)	2.6	340	0.125~14.9	/	[78]
Beryllon II	3.80	/	0.10~32.40	/	[79]
Beryllon II-Al(III)	5.6~7.2	400~420	0.2~41.2	87.0	[80]
m-Acetylchlorophosphonazo (CPA-mA)	4.1	408	0.50~30.0	52.7	[81]
m-carboxychlorophosphonazo(CPA-mK)	4.1	410	0.5~35.0	104	[82]
Fast red VR(FRV)	3.52	287.0	0.1~8.0	7.1	[83]
Titan yellow(TY)	4.1	/	0.1~5.0	12.7	[84]
Dibromochloro-arsenazo-Al^{3+}	5.0~7.0	405~420	2.5~50.0	123.4	[85]
Arsenazo-DBS		400~420		77.0	[86]
Arsenazo-DBN-Al(III)	5.3~7.0	420~440	0.34~41.71		[87]
Resorcinol yellow(RY)-OP	2.35		0.02~4.0	10.4	[88]
Arsenazo I	3.29	400	0~18	60	[89]
Thorin			1.8~4.7	52.0	[90]
Arsenazo M (AA M), Chlorophosphonazo III (CPA III), Chlorosulphonphenol S (CSP S)	3.4~4.0	470	0~4.8	18.5	[91]
Eriochrome black T(EBT)	4.1	375	0~15.0	39.0	[92]
Other chromophores					
Alcian blue 8GX (ABGX)	7.24	398	0.1~3.0	8.2	[93]
Tetra-substituted sulphonated aluminum phthalocyanine (AlS_4Pc)	3.0	413	0.050-2.0 (HSA)	12.7	[94]
PsbMo heteropoly blue (PSbMo)	Acidic medium	470	0~6.0	21.0	[95]
Morin-CTMAB	7.8~8.0	305/610	0.075~10.0	66.0	[96]
Pyrocatechol violet (PV)-Triton X-100	1.4~2.0	341	0~8.0	0.05	[97]
$K_3[Fe(CN)_6]$	2.7	351	0~12((HSA)	100	[98]
Nanoparticles					
Colloidal silver chloride	3.0~9.0	398, 475	0.001~0.4	8	[99]
Reagents without absorption in VIS region					
Sodium lauryl sulfate (SLS)	1.81~4.1	470	/	/	[100]
Sodium dodecane sulfonate (SDS)	1.98	470	0.023~6.0	23.7	[101]
Sodium dodecylbenzene sulfonate- Thorium	6.37~6.59	340~370	0.15~1.0(HSA)	14.4	[102]

Table 3 Determinations of calf thymus DNA with its enhanced RLS signals on chromophores

Chromophores	pH	RLS peak (nm)	Linear ranges ($\mu g \cdot mL^{-1}$)	LOD (3σ; $ng \cdot mL^{-1}$)	Reference
Cation porphyrins & phthalocyanines					
Tetra-amino aluminum phthalocyanine (TAA1Pc)	6.0	400	0~0.25	1.4	[103]
Quinone-imines					
Brilliant cresol blue (BCB)	4.1~9.0	364	0.12~4.70	118.0	[104]
Brilliant cresyl blue	11.0~11.5	347	0.08~1.0	2.3	[105]
Neutral Red (NR)	7.63	535.0	0~1.5	10.32	[106]
Neutral Red (NR)	5.0~7.0	335.0	0~0.6	12.8	[107]
Azur A (AA)	3.29	398	0~2.0	10.9	[108]
Azur B (AB)	1.98~2.56	359.7	0~3.0	8.7	[109]
Methylene blue (MB)	5.5~7.5	350	0.2~1.4	1.5	[110]
Janus green B (JGB)	6.37	416.0	0~3.5	9.9	[111]
Basic triphenylmethanes					
Methyl violet (MV)	7.51	651.0	0~3.0	404.5	[112]
Crystal Violet	5.03	666.0	0~4.5	36.8	[113]
Brilliant Green (BG)-CTMAB	10.4~11.8	398.0	0~1.2	3.9	[114]
Methyl green (MG)-CTMAB	6.9~7.2	414.0	0.025~1.0	7.8	[115]
Rosaniline	10.5~10.8	485.0	0~1.0	14.2	[116]
Butyl rhodantine B	1.1	335.0			[117]
Drugs					
Dequalinium chloride (DC)	7.0	362	0.04~10	6.2	[118]
Thiamine Hydrochloride (TH)-CTMAB	/	406	0.02~10	11.0	[119]
Berberine-CTMAB	7.30	416.5	0.0075~75	2.1	[120]
Berberine	2.0~2.8	308	0~600	20	[121]
Other chromophores					
Pd(II)/5-Br-PADAP)	5.9~7.5	582	0~5.0	44	[122]
Pd(II)/2-(2-thiazolylazo)-5-dimethylamino-benzoic acid (TAMB)	5.9	675	0~3.5	22	[123]
Alcian blue 8GX	7.54	398.8	0.25~2.0	7.1	[124]
Histone	4.5~6.5	551	0.05~1.5	2.0	[125]
Morin-CTMAB	7.30	462.2	0.0075~10.0	3.4	[126]
Morin-CTMAB	7.30		0.025~5.0	16.7	[127]
Acridine red (AR)-CTMAB	6.40~7.10	555	0.05~1.2	8.53	[128]
Cetyl Trimethyl Ammonium Bromide (CTMAB)	2.21	470	0.05~2.5	4.9	[129]
CTMAB	8.5	414	0.025~20.0	8.3	[130]
CTMAB, Cetylpyridine bromide (CPB)	2.0~8	470	0~3.0	13.3, 7.30	[131]
Cetylpyridine bromide (CPB)		310~400	0.005~50	4.3	[132]
Eu-Trioctyl phosphine oxide (TOPO)	8.69	348, TIR-RLS	0.002~2.5	0.16	[133]
Al^{3+}	2.21	291.0	0~5.0	6.1	[134]
HCl	0.1 $mol \cdot L^{-1}$	310	0.06~100	18.0	[135]
H_2SO_4	0.1 $mol \cdot L^{-1}$	343	0.08~70	17.8	[135]
HNO_3	0.1 $mol \cdot L^{-1}$	310	0.1~100	18.4	[135]
AgCl sol	4.0	398	0~20	0.65	[136]

1.1.4.3 Molar ratio of the interacting components

It was found that enhanced RLS signals are generally available at low ionic strength and high molecular ratio of chromophores to biomolecules. Under such conditions, UV and fluorescence detections are insensitive, but the long-range assembly of dyes along the biomolecular surfaces can induce strong RLS signals since the change of biomolecular conformations induced by the large concentrated amount of dyes. For example, superhelical helixes of nucleic acids could be induced when the long-range assembly of positively charged porphyrin occurs, displaying strong enhanced RLS signals near the Soret absorption region[137]. With decrease in the molar ratio, which is very common in the study of pharmacology, RLS signals begin reducing, indicating chromophores intercalate into the interior of the double stranded structure. Thus, the change of the RLS signals could be used to assign the binding mode of the chromophores with nucleic acids, and correspondingly, the chromophores act as molecular structural probes of nucleic acids.

1.1.4.4 Molecular absorption in the medium

Unlike conventional light scattering techniques in which the absorption wavelength region of chromophores is avoided, RLS measurements focus on this portion of the electromagnetic spectrum. Since the molecular absorption species should absorb both the excitation and the light scattering, the available RLS signals corresponding the absorption band are reduced, inducing distortion of the real RLS spectrum[138]. Thus, similar to the corrections needed in fluorescence spectroscopy for primary and secondary absorption processes, Pasternack and co-workers proposed that RLS spectra need to be corrected for the absorption of the incident and the scattered light in order to extract useful information from the spectroscopy experiments on aggregates of the chromophores[139]. The basic theory of the correction is to combine the extinction and RLS measurements for the same samples by introducing a correction factor to compensate for the absorption loss of the enhanced light scattering signals[22,139,140].

1.1.4.5 Sensitivity of RLS methods

The sensitivity of RLS methods depends on the properties of the molecular structures of the interacting components, and the environmental conditions. As displayed above, the RLS signals resulting from the interactions of chromophores with proteins or nucleic acids are strongly dependent on the molecular structures, pH and ionic strength of the aqueous medium. If the size is the main factor responsible for the enhanced RLS signals, the enhanced RLS intensity is proportional to the molecular weight of proteins [93]. Besides these factors, the concentrations of the chromophores used for the determination are dependent also [141,142]. In addition, some interactions, especially the interaction of quinone-imines with nucleic acids, display several RLS peaks. The sum of the RLS intensities at these peaks can be used for the determination of nucleic acid, and the sensitivity can be improved [143].

Although the cmc value of a surfactant could be determined by measuring the RLS signals of micelle in aqueous medium[144], it has been proved that cation surfactants are able to sensitize the interactions between chromophores and DNA[114]. The RLS intensities of chromophores–surfactants–buffer are easily affected with pH variation, so pH control should be specially considered when discussing these interactions of chromophores–DNA–surfactants. The interaction of CTMAB–DNA, for example, has strongly enhanced RLS signals at pH 2.21, but the signals decrease steeply when pH is higher than 4.5[129]. In addition, ethanol was also found to have

sensitizing effect on the determination of proteins with Titan Yellow[84].

1.1.5 RLS at liquid/liquid interfaces

As stated above, RLS technique ($\lambda_{em} = \lambda_{ex}$) is simple, sensitive, and has broadly practiced in analytical chemistry. However, the technique has poor selectivity, and its tolerance of coexisting foreign substances is scarcely at the same level as the analytes. The reason is that RLS signals of scatterers are related to the absorption features of the medium, size, shape, concentration, and refractive index compared to the scatterers' environments [21,145~147]. Thus, particles or colloidal scatters of coexisting foreign substances can exert influences significantly[22~24]. Surfactants, metal ions, amino acids, and sugars have different interference on the RLS methods since they can interact with the biomolecules through electrostatic attraction or hydrophobic interactions, thus measures should be taken, such as adjusting pH value[129], diluting the sample[94] or using standard addition method[67] to reduce the effect of foreign materials or the background in turbid medium.

Since RLS technique is established on the basis of the fluctuation of the refractive indexes in an aqueous solution where a steep change occurs between the refractive index of the inner scattered particles and that of their outer atmosphere[21,145], a total internal reflected resonance light scattering (TIR-RLS) technique has been developed[62]. It has been proved that TIR-RLS holds following advantages: (1) TIR-RLS will easily bring about a sharp fluctuation of refractive indexes at the interfacial region due to the formation of an evanescent field, leading to much stronger enhanced RLS signals. The liquid/liquid interface is somewhat similar to an enlarged surface of a scattered particle and the refractive indexes play the same role as they do in a bulk phase according to Eq. (1); (2) the analytes can be adsorbed to the oil/water interface to acquire a good separation of the analytes with coexisting foreign substances, during which enrichment of the analytes at the oil/water interface also occurs, and high selectivity and sensitivity can be expected as well[62]. Compared to the methods based on RLS measurements and spectrofluorometry[148], the sensitivity of TIR-RLS methods for clinical drugs is about 100–1000-fold higher[61,62,102]. In addition, it has been found that enhanced RLS signals of the interaction of nucleic acids with dyes can be observed only in a medium of low ionic strength in an bulk aqueousmedium, while, however, the interaction can proceed or enhanced RLS signals can be observed in a medium of ionic strength higher than 0.2 M in the TIR-RLS system[133]; (3) hosts and guests with immiscible property can encounter and interact at an oil/water interface, thus corresponding amphiphilic species can be separated from the bulk phases, adsorbed and enriched by the oil/water interface [62,149], leading to that the recognition and interaction of hosts and guests with immiscible property can be carried out easily at the liquid/liquid interfaces that are lower than 200 nm in thickness[66,150]; (4) oil-soluble chromophores can be used as reagents conveniently owing to the existence of the oil phase [102]; (5) the interfacial region presents a good chance to study orientation of molecules by means of polarized incident light beams as a result of the relatively simplex components in it. Therefore, TIR-RLS technique is not only applied to analytical purposes, but also shows high promise in the characterization of the molecular assembly, recognition, drug extraction and transport at liquid/liquid and liquid/vapor interfaces.

1.1.6 Future of RLS technique

As a new spectral analysis technique, the light scattering measurements by using a common spectrofluoro-

meter are very simple, and sensitive. This technique is generally coupled to other spectral analysis techniques such as absorption, fluorescence and CD, and can compensate for the drawbacks of spectrophotometric and spectrofluorometric measurements. Hyperchromism and hypochromism are generally found in the interaction between chromophores with proteins or nucleic acids, thus the spectrophotometry or spectrofluorometry based on them have low sensitivities. On the contrary, methods based on measuring enhanced RLS signals that are generally related to hyperchromism and hypochromism are much more sensitive. Furthermore, RLS methods could be established in a turbid system without UV-Vis absorption properties, extending the usefulness of reagents in the quantitative analysis of medicines, proteins and nucleic acids [93,130,135]. Even though we believe that there still is an extensive space for further development of light scattering techniques in terms of analytical proposes.

1.1.6.1 RLS as a technique for spectral assignment

RLS technique has been successfully to assign the assembly and aggregate species, and applied for analytical purposes, but as a spectral technique, it is much better if it could be used for fine spectral assignment [37]. Especially, the relationship of the RLS spectra with the molecular structure should be further assigned. For example, Methylene Blue (MB), Azure A(AA), Azure B(AB) and Lauthis Violet (LV) have similar molecular structure, but the RLS features displayed molecular structural dependent when interacting with nucleic acids are greatly diferent, and thus possibly involving in different interaction mechanism [108,109,151]. According to semi-empirical molecular calculation, the different interaction mechanism mainly results from the substitute groups and the coplanar structures. The structure dependence of the RLS signals can be seen also from the distribution variation of the charge density of basic triphenylmethane dyes before and after these dyes forming ion associates with iodide anion and metal cations[152,153].

1.1.6.2 Developing Raman light scattering technique in UV-Vis region

Bedsides RLS signals ($\lambda_{em}=\lambda_{ex}$), other light scattering signals can be detected by using a three-dimensional (3D) functioned spectrofluorometer including second-order light scattering (SLS, $\lambda_{em}=2\lambda_{ex}$), anti-second-order light scattering (ASLS, $\lambda_{em}=0.5\lambda_{ex}$) and Raman light scattering in the assembly of chromophores on the template of DNA[154]. The wavelength of light scattering signals has linear relationship with that of incident light beams. SLS and ASLS signals are weaker than RLS ones for same scatterers, and the sensitivity of SLS and ASLS methods are lower than that of RLS method[154]. Raman light scattering signals are always found along RLS, SLS, and ASLS. The weak enhanced Raman light scattering signals in UV-Vis region open new avenues of technology that could designate Raman light scattering signals in UV-Vis region. It is technically possible by using a variable-angle controlled spectrofluorometer to measure the Raman light scattering signals in UV-Vis region under a high sensitivity function mode.

1.1.6.3 Developing new clinical instrument for cancer diagnosis

Compared to the diagnostic techniques of high energy X-rays and expensive magnetic resonance images, light rays in UV-Vis region is much safer and cheaper for diagnosis of tissue problem. Thus, light scattering image techniques have begun to find applications in clinical test and diagnostics. One big finding is that cancer cells generally have high density of cell matrix, and have stronger light scattering signals than normal cells, so back