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CHEMOENZYMATIC FUNCTIONALIZATION OF RIBONUCLEIC ACID WITH AZOBENZENE CHROMOPHORES

Sucharita Roy,[1] Ramaswamy Nagarajan,[2] Peichuan Wu,[2] Ke Yang,[2] Ferdinando F. Bruno,[3] Virinder S. Parmar,[1] Sukant K. Tripathy,[2,†] Jayant Kumar,[2,*] and Lynne A. Samuelson[3,*]

[1]Department of Chemistry, University of Delhi, Delhi 110 007, India
[2]Departments of Chemistry and Physics, Center for Advanced Materials, University of Massachusetts—Lowell, Lowell, MA 01854
[3]Natick Soldier Center, U.S. Army Soldier & Biological Chemical Command, Natick, MA 01760

This paper is dedicated to the memory of Sukant K. Tripathy, our dear friend and colleague, whose inspiration, creativity, and achievements in science will always live on.

ABSTRACT

In recent years, biological molecules have brought about a renaissance in the development of novel responsive materials. An example of this is the development of new photoresponsive materials for the artificial regulation of chemical and biological systems. Towards this we have developed a novel enzymatic synthetic approach for covalent attachment of photoresponsive units into the RNA backbone. This involves a lipase catalyzed acylation of the 2' hydroxyl group in the ribose sugars in the RNA molecule to incorporate photo-isomerizable azobenzene groups into the RNA strands. A reverse micellar approach was used for this RNA functionalization to maintain the solubility of the nucleic acid as well as to limit the preferred hydrolysis reaction in aqueous media. The azobenzene groups incorporated in the RNA molecule show photo-isomerization behavior and can serve as optical 'handles' for the manip-

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ulation of the conformation of RNA. This modification of RNA using covalently attached chromophores or fluorophores is a generic approach that can be extended to other biomacromolecular matrices leading to new opportunities for biophotonic device applications.

Key Words: Ribonucleic acid; Reverse micelles; Azobenzene; Photo-isomerizable

INTRODUCTION

Ribonucleic acids are a multifaceted group of molecules, being involved in all steps of protein synthesis by storing the genetic information (mRNA) or by carrying amino acids onto the ribosomes (tRNA). There has been an increased interest in recent years for exploring different pathways to monitor the conformational dynamics of the RNA's. The precise mapping and folding of the RNA secondary and tertiary structures are of prime importance for the understanding of the RNA functions. Various chemical and enzymatic footprinting methods are known for following the conformational changes such as chemical modification [1], oligonucleotide hybridization [2], UV crosslinking [3] and more recently, synchrotron hydroxyl radical foot printing [4], among others. Several studies have investigated fluorescence techniques to monitor the protein folding and unfolding, with probes either covalently attached or non-covalently associated [5]. In particular, azobenzene derivatized macromolecules have been widely investigated as photo-responsive triggers in regulation of enzyme activity [6], which provides a general approach to utilize biocatalysts as light controlled 'on-off' switching systems. The photoregulation of enzymes involves either using photochromic materials that act as inhibitors for the enzyme active site specifically in one of their photochromic configurations or chemical derivatization of the enzyme active site by substituents capable of blocking the biocatalyst activity in specific configurations [7]. A detailed investigation of photochromism and conformational as well as light induced effects in polypeptides such as poly(L-glutamic acid) [8] and poly(L-lysine [9] having various contents of azobenzene units in the side chains has also been reported. Other light induced reversible variations in the physical and chemical properties of azobenzene containing polymers include conformational changes, variations in viscosity and solubility, photo control of pH, photoregulation of binding/releasing of drugs, photomechanical effects [10-12] and potential data storage materials [13, 14].

Recently, attempts have been made to develop photo-responsive systems in relation to nucleic acids, which are envisaged as an attractive methodology to regulate the interactions between the oligonucleotides and other molecules by photo-irradiation. If the conformation of the nucleic acids can be controlled simply by photo-exposure without changing the pH or ionic strengths, various applications

in vitro and *in vivo* are promising possibilities. A recent report on modification of oligonucleotides involves the tethering of the azobenzene residue to the 2' position of uridine [15]. On photo-irradiation of these modified oligonucleotides, the melting temperature of the duplex between the oligonucleotide and the complementary DNA significantly changes due to *cis-trans* isomerization of the azobenzene. Azobenzene groups have also been introduced into the main chains [16], as well as the side chains [17] of the oligonucleotides. These azobenzene residues have been primarily introduced through *para* substituted functional groups ($-NH_2$, -COOH and –OH) since these substituted azobenzenes are commercially available and are easy to synthesize. It has been known that rapid thermal isomerization of *para* substituted *cis* azobenzene is attributed to the electronic effects of the substituents that weakens the N=N bond. Thus it is expected that the thermal stability would be improved if substituents were attached to the *meta* position of azobenzene. Asanuma and co-workers [18] introduced *m*-aminoazobenzene into the side chain of the oligonucleotides to inhibit the thermally induced *cis-trans* isomerization of azobenzene in order to achieve strict photo-regulation without thermal isomerization.

However, in each of these cases the attachment of the azo groups was carried out prior to the nucleotide synthesis. To date there has been no attempt to tether the azobenzene groups directly to a genomic RNA/DNA. It is well documented that as a biomolecule folds, a covalently attached or non-covalently associated chromophore is typically exposed to a different microenvironment thus altering its fluorescence properties. Here we have investigated the possibility of a unique photoresponsive assay involving the anchoring of azobenzene photoresponsive units directly to RNA for optical monitoring of the structural or conformal changes in the biomacromolecule. Towards this goal, we have designed an enzymatic route for the functionalization of RNA in dynamic reverse micellar media.

In recent years, reverse micellar enzymology has received a great deal of attention. This field involves small nanometer sized dispersions of aqueous/polar materials in lipophilic organic solvents formed by the action of surfactants as a medium for hosting numerous reactions. These systems have many advantages, including thermodynamic stability, low water content (water drives ester hydrolysis thus limiting the extent of esterification), large interfacial area that allows a higher degree of enzyme-substrate interaction, ease of preparation and solubilization of biopolymers like nucleic acids in lipophilic organic solvents [19]. Lipase catalysis of esterification reactions has been investigated in reverse micelles [20-22], the most frequent examples are that of the esterification of fatty acids and alcohols. Transesterification reactions have also been investigated [23], but it has been shown that reverse hydrolysis reactions occurred to a considerable extent in these cases. The rate of the enzymatic reaction in reverse micelles is reported to depend on many factors such as surfactant concentration, the water to surfactant molar ratio (w_o), temperature, concentration of buffer and the type of counter ions present [24].

EXPERIMENTAL

Materials and Methods

Ribonucleic acid (from yeast source) was obtained from Calbiochem, while porcine pancreatic lipase (PPL, EC 3.1.1.3, Type II) was obtained from Sigma Chemical Co. (St. Louis, MO). 4-(Phenylazo) benzoic acid (purity 98%) and dioctyl sodiumsulphosuccinate (AOT, purity 98%) were purchased from Aldrich Chemicals, Inc., Milwaukee, WI. All other chemicals were of reagent grade or better and were used as received. The RNA solutions were checked for protein contamination prior to use by measuring the UV-vis absorbance at 260 nm as well as 280 nm. Sterile glassware, as well as buffers, (100 mM sodium phosphate pH 7.0 and 40 mM sodium tetraborate buffer, pH 9.0) were used for the preparation of the RNA solutions. Gel electrophoresis was carried out using 6% trisborate–ethylenediaminetetraacetic acid (TBE)-urea polyacrylamide gels with TBE as running buffer.

Synthesis of RNA-Azo Chromophore Complex

A 50 mM solution of the surfactant AOT in isooctane was prepared, and azo benzoic acid was dissolved in this medium (100 mM) with sonication. Lipase was added to this media, followed by the solubilization of the nucleic acid in sodium tetraborate buffer and subsequent injection into the micellar solution. The final water content of the hydrocarbon phase was expressed in terms of the parameter w_0 {[H_2O]/[AOT]}. A clear monophasic reverse micellar media was obtained and the reaction was monitored through gel electrophoresis, UV-vis spectroscopy and circular dichroism polarimetry. The reaction was worked up by 'bursting open' the micelle by adding excess buffer of high ionic strength. The final product was isolated by purification through molecular weight cutoff membranes (Centricon), followed by subsequent lyophilization.

Characterization

Dynamic light scattering measurements were performed using a Brookhaven instrument (Model SG-7B Rigaku Denki, Japan) with a homemade goniometer and a scattering cell chamber. The excitation source was a linearly polarized 35 mW He-Ne laser (Melles-Griot, Model No 05-LHP-927) operating at 632.8 nm, which was housed on the goniometer fixed arm. The detection unit consisted of an Avalanche Photo Diode (RCA make, Model # SPCM-100), a discriminator and a pre-amplifier mounted on the goniometer rotating arm. The UV-vis spectra of RNA and the RNA–azo complex were recorded on a Perkin-Elmer Lambda-9 spectrophotometer. The circular dichroism (CD) polarimetry spectra were obtained using a JASCO CD Spectrometer J-720. The FTIR Spectra were

recorded on a Perkin-Elmer 1720 X series. The gel electrophoresis studies were visualized under a UV-transilluminator (302 nm) after staining with ethidium bromide.

RESULTS AND DISCUSSION

A schematic of the reaction leading to the RNA-azo chromophore complex is illustrated in Figure 1. As shown, the azo group is incorporated into the RNA backbone through an esterification reaction between the acid moiety in the azobenzene and the 2' hydroxyl group present in the RNA ribose sugar. The micellar sizes were determined by the dynamic light scattering experiments and the results show that the apparent micellar sizes were of the order of nanometers. It was found that the micellar size increased with the w_o value and the diameter sizes ranged from 8.0 nm–11.3 nm. The calculations in each of these experiments were done on the assumption of the micelle being a conventional spherical particle.

A typical UV- vis spectrum of the azobenzene incorporated RNA is shown in Figure 2. There are two major absorption bands at 258 nm and 323 nm, the former one is assigned to the nucleic acid bases while the latter to the *trans* form of the azobenzene. Upon UV light irradiation (300nm<λ<400 nm), the *trans* form isomerized to the *cis* form as shown by the decrease in the intensity of the *trans* band accompanied by a corresponding increase in the *cis* band. This photo-isomerization occurred in the esterification reaction catalyzed by PPL at w_o=9 and this data agrees well with previously reported results of photoisomerization of azobenzene linked oligonucleotides.

The CD studies also show bands in the region of 330-350 nm (Figure 3) that indicate the presence of the azo dye in the complex since these bands coincide with the absorption peak of the azo dye. This peak in the CD spectral data can only be explained on the fact that the functionalization of the RNA with the azo

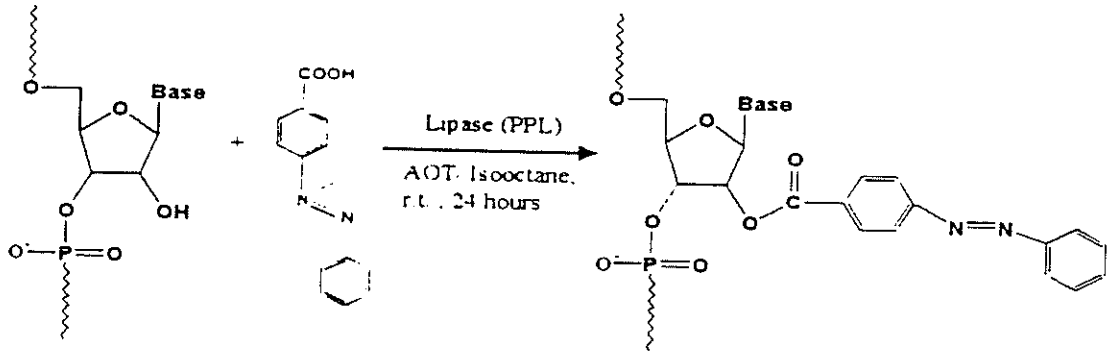

Figure 1. Schematic of azo functionalized RNA formation.

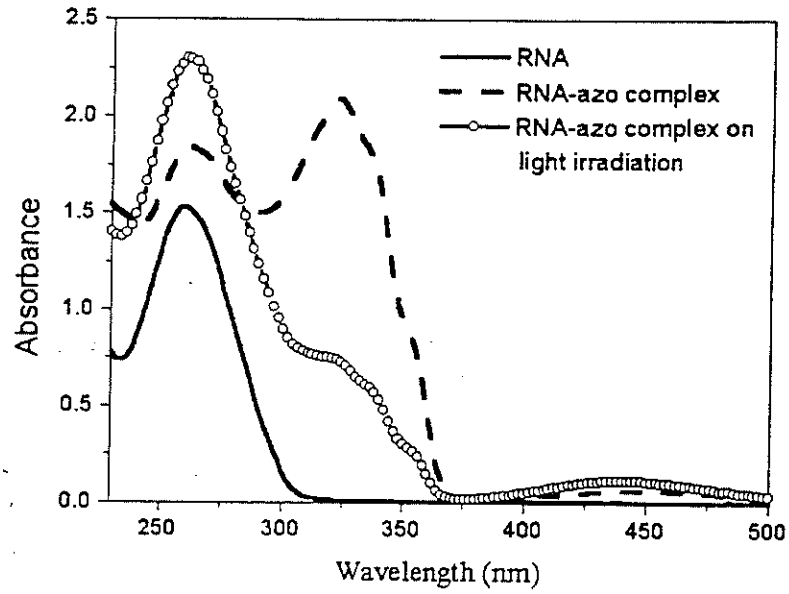

Figure 2. UV-vis data of RNA functionalization by PPL on excitation with UV light.

dye may result in the azo dye adopting a 'handedness' along the RNA. Similar results were also obtained at $w_{o=}$14 showing a positive ellipticity in the region corresponding to the absorption of the azo dye.

The formation of the ester between the 2' hydroxyl group of the ribose moiety and the 4-(phenylazo) benzoic acid was confirmed by the appearance of peaks at 1712 and 1716 cm^{-1} in the FTIR spectrum of the modified RNA as compared to

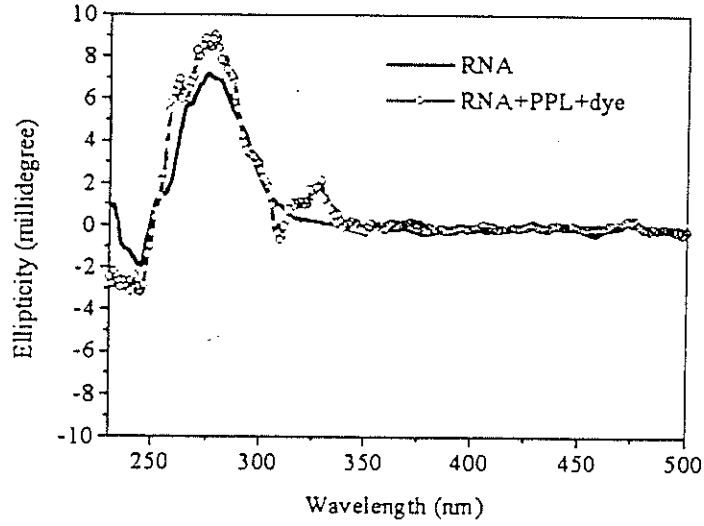

Figure 3. CD spectra of RNA-azo complex functionalized using PPL at w_0=9, pH 7.0.

the azobenzene carboxyl peak at 1680 cm^{-1} and the RNA peaks (carbonyl region) ranging between 1692-1698 cm^{-1}. Guided by the literature reports [19] that nucleic acids may be solubilized in reverse micellar media at a higher pH, the effect of pH on the esterification reaction was investigated. On carrying out the esterification reaction at pH 9, $w_{o=}$22 it was observed that the functionalized RNA partitioned into the isooctane phase. The presence of the azo functionalized RNA in the isooctane layer was established by the UV–vis and CD spectral data as shown in Figures 4 and 5. The CD spectral data indicate the presence of the azo dye peak while a noticeable change in the RNA peak is also observed which could be attributed to a change in the conformation of the RNA.

The gel electrophoresis results show the native RNA, the reaction mixture and the control experiment (RNA and the azo dye without lipase) in wells 1, 2, and 3, respectively. As can be seen in Figure 6, wells 1 and 3 have a fluorescent spot signifying the ribonucleic acid on staining with ethidium bromide (EtBr) solution while there is no visible spot for the same in well 2 (reaction product). This disappearance can be explained based on the covalent attachment of the azo dye leading to either a change in the electrophoretic mobility of the nucleic acid or a distortion of the RNA helix in a manner, which impedes the intercalation of the EtBr dye. It was interesting to note the appearance of a spot for the simultaneous control experiment, which was carried out in the absence of lipase. This indicated that the unreacted azo chromophore, even if intercalating with the RNA helix (in the absence of reaction), did not interfere with the intercalation of the EtBr, which is used to visualize the nucleic acids. Thus it can be deduced that the nucleic acid

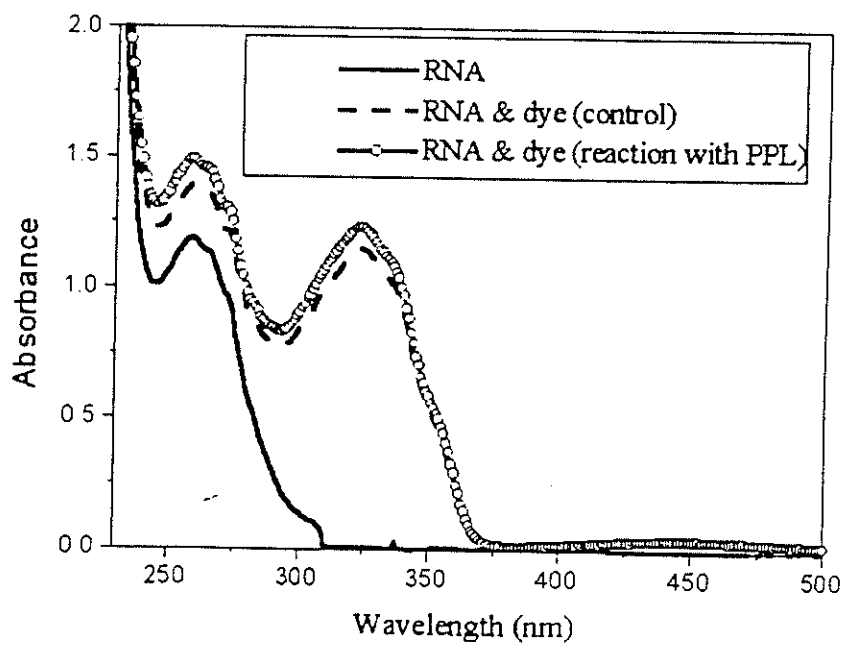

Figure 4. UV-vis spectra of azo functionalized RNA on reaction with PPL at w_0=22, pH 9.0.

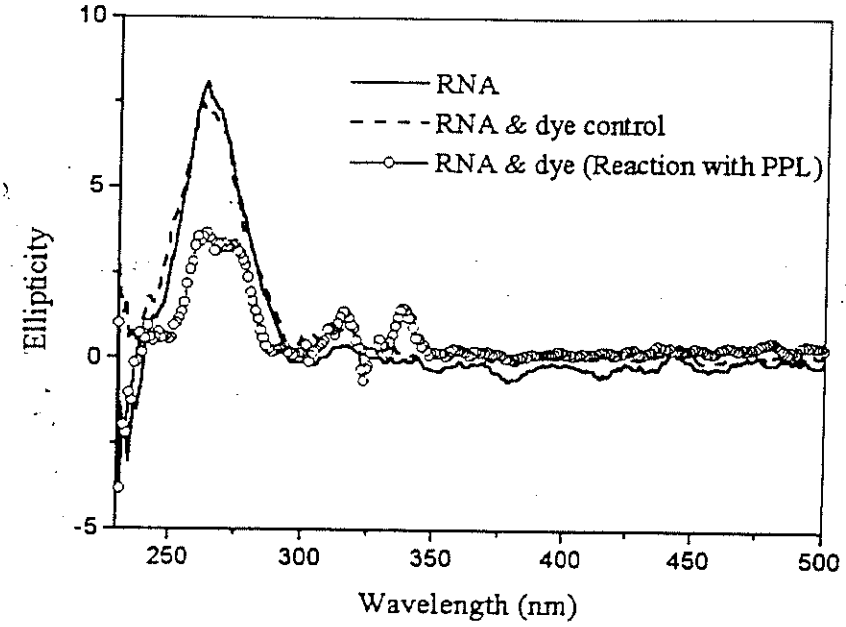

Figure 5. CD spectral data of azo functionalized RNA on reaction with PPL at w_0=22, pH 9.0.

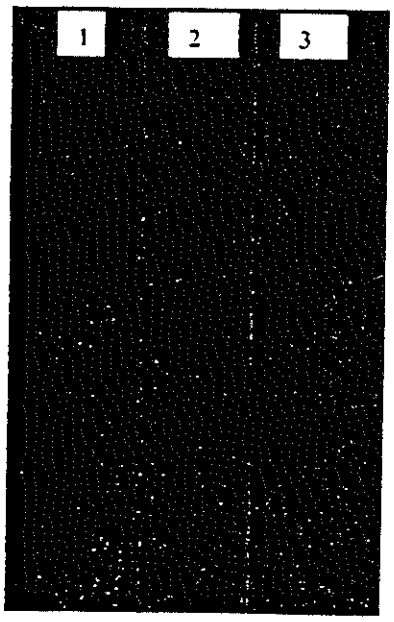

Figure 6. Gel electrophoresis results of esterification reaction on RNA catalyzed by PPL at w_0=22, pH 9.0 in the isooctane layer where the wells indicate: (a) native RNA; (b) RNA and azo dye in the presence of lipase; (c) RNA and azo dye in the absence of lipase (control).

on reaction with azo dye in the presence of lipase has undergone some conformational change, which cannot be due to the mere intercalation of the azo dye.

These results indicate that the method of chromophore functionalization of the nucleic acid complex can alter the solubility of the complex, thus providing us the opportunity to process these post functionalized biopolymers as potential information storage devices (surface relief gratings). Further experiments are still in progress to establish the nature of the covalent attachment (ester or amide formation) of the azo dye to the RNA.

CONCLUSION

Our results show that a biopolymer (RNA) has been functionalized with an azo chromophore by an enzyme catalyzed acylation reaction. The reverse micellar media offers the benefit of maintenance of the conformational integrity of the RNA secondary structure, on encapsulation in the biocompatible microaqueous neutral buffer environment. The photoactive group incorporated in the RNA molecule opens new avenues for the investigation of RNA conformational switching through the photodynamic behavior of the azo chromophore as well as for the fabrication of versatile functional biomacromolecular matrices.

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REFERENCES

1. Banerjee, A R , Turner, D H The Time Dependence of Chemical Modification Reveals Slow Steps in the Folding of a Group I Ribozyme. Biochemistry **1995**, *34*, 6504-6512
2. Zarrinkar, P P, Wang, J , Williamson, J R. Kinetic Intermediates in RNA Folding. Science **1994**, 265(5174), 918-924.
3. Downs, W.D , Cech, T R Kinetic Pathway for Folding of the Tetrahymena Rbozyme Revealed by Three UV-Inducible Crosslinks. RNA **1996**, *2*, 718-732.
4. Sclavi, B.; Sullivan, M., Brenowitz, M.; Woodson, S.A. RNA Folding at Millisecond Intervals by Synchrotron Hydroxyl Radical Footprinting. Science **1998**, *279*, 1940-1943.
5. Silverman, S. K.; Cech, T. R. RNA Tertiary Folding Monitored by Fluorescence of Covalently Attached Pyrene. Biochemistry **1999**, *38*, 14224-14237.
6. Hamachi, I.; Hiraoka, T.; Yamada Y.; Shinaki, S. Photoswitching of the Enzymic Activity of Semisynthetic Ribonuclease S' Bearing Phenylazophenylalanine at a Specific Site. Chem. Lett. **1998**, 537-538.

7. Willner, I.; Rubin, S.; Riklin, A. Photoregulation of Papain Activity Through Anchoring Photochromic Azo Groups on the Enzyme Backbone. J. Am. Chem. Soc. **1991**, *113*, 3321-3325.
8. Houben, J.L.; Fissi, A.; Bacciola, D.; Rosato, N.; Pieroni, O.; Ciardelli, F. Azobenzene-Containing Poly(L-glutamates): Photochromism and Conformation in Solution. Int. J. Biol. Macromol. **1983**, *5*, 94-100.
9. Fissi, A.; Pieroni, O.; Ciardelli, F. Photoresponsive Polymers: Azobenzene-Containing Poly (L-Lysine). Biopolymers **1987**, *26*, 1993-2007.
10. Smets, G. Photochromic Phenomena in the Solid Phase. Adv. Polymer Sci. **1983**, *50*, 17-44.
11. Montagnoli, G.; Pieroni, O.; Suzuki, S. Control of Peptide Chain Conformation by Photoisomerizing Chromophores: Enzymes and Model Compounds. Polym. Photochem. **1983**, *3*(4), 279-294.
12. Irie, M. in *Photophysical and Photochemical Tools in Polymer Science*, Winnik, M.A, Ed. Reidel Publishing Co.: Dordrecht, 1986; 269-291.
13. Viswanathan, N.K.; Kim, D.Y.; Bian, S.; Williams, J.; Liu, W.; Li, L.; Samuelson, L.; Kumar, J.; Tripathy, S.K. Surface Relief Structures on Azo Polymer Films. J. Mater. Chem. **1999**, *9*, 1941-1955.
14. Alva, K.S.; Lee, T.S.; Kumar J.; Tripathy, S.K. Enzymatically Synthesized Photodynamic Polyaniline Containing Azobenzene Groups. Chem. Mat. **1998**, *10*(5), 1270-1275.
15. Asanuma, H.; Yoshida, T.; Ito, T.; Komiyama, M. Photo-Responsive Oligonucleotides Carrying Azobenzene at the 2'-Position of Uridine. Tetrahedron Lett. **1999**, *40*, 7995-7998.
16. Yamana, K.; Yoshikawa; A. Nakano, H. Synthesis of a New Photoisomerizable Linker for Connecting Two Oligonucleotide Segments. Tetrahedron Lett. **1996**, *37*, 637-640.
17. Asanuma, H.; Ito, T.; Komiyama, M. Photo-Responsive Oligonucleotides Carrying Azobenzene in the Side-Chains. Tetrahedron Lett. **1998**, *39*, 9015-9018.
18. Asanuma, H.; Liang, X.; Komiyama, M. *meta*-Aminoazobenzene as a Thermo-Insensitive Photo-Regulator of DNA-Duplex Formation. Tetrahedron Lett. **2000**, *41*, 1055-1058.
19. Imre, V. E.; Luisi, P. L. Solubilization and Condensed Packaging of Nucleic Acids in Reversed Micelles. Biochem. Biophys. Res. Commun. **1982**, *107*(2), 538-545.
20. Tsai, S-W.; Lu, C-C.; Chang, C-S. Enantioselective Esterification of Naproxen in AOT/Isooctane Reversed Micelles by Lipase. J. Chin. Inst. Chem. Eng. **1996**, *27*(3), 201-204.
21. Yang, C-L.; Gulari, E. Enzymic Esterification of Diols in Reverse Micellar Media. Biotechnol. Prog. **1994**, *10*, 269-276.
22. Hayes, D. G.; Gulari, E. 1-Monoglyceride Production from Lipase-Catalyzed Esterification of Glycerol and Fatty Acid in Reverse Micelles. Biotechnol. Bioeng. **1991**, *38*, 507-517.
23. Abraham, G.; Murray, M. A.; John, V. T. Interesterification Selectivity in Lipase Catalyzed Reactions of Low Molecular Weight Triglycerides. Biotechnol. Lett. **1988**, *10*, 555-558.
24. Fendler, J. H. in *Membrane Mimetic Chemistry*, John Wiley & Sons: New York 1982.