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Bioconjugation of Hepatitis B antigenic peptide with polymeric carriers through various carbodiimide chemistry*

[Hepatit B antijenik peptidinin polimerik taşıyıcılara karbodiimid kimyası aracılığıyla biyokonjugasyonu]

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*In the loving memory of Prof. Dr. Mamed MUSTAFAEV, Founder Head of Yıldız Technical University BioengineeringDepartment, precious man of science.

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ABSTRACT

Aim: The purpose of improving function-specific bioconjugates using polyacrylic acid (PAA), the 95-109 amino acid series of Hepititis B surface antigen epitopes, and different polyelectrolytes such as Poly (vinyl pyrrolidone-co-acrylic acid) (VPAA) copolymer is to increase the immunogenity of the antigen peptide. The aim of this study is to use the bioconjugates, obtained from the said conjugation reactions, as models in order to improve new generation vaccinations.

Methods: The bioconjugates were synthesized using a water soluble carbodiimide 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide via different carboxyl activation mechanisms. After choosing the most effective conjugation method, two different polyelectrolytes, and initial molar ratios were used in conjugation reactions to show the efficiency of the method. In addition, the synthesized bioconjugates were analyzed using Size Exclusion Chromatography and Fluorescence Spectrometer.

Results: Chromatographic analyses showed that the best coupling method between Polyelectrolytes and the peptide molecule was Method 3, and the bioconjugation reactions using this method proved that when the peptide concentration increases, the peptide molecule directly binds with polyelectrolytes, and the molecular weight of the resultant bioconjugates increases proportionally as shown in the chromatograms.

Conclusion: The chosen activation method provides a strong covalent bond between the peptide and polyelectrolyte molecules, and approaches developed to modify the immunogenecity of antigens opens perspectives for creating new vaccines, diagnostic, pharmaceutical, and biotechnological preparations.

Keywords: bioconjugation, synthetic peptides, polyelectrolytes, water-soluble carbodiimide

There is no conflict of interest between the authors.

ÖZET

Amaç: Hepatit B virüsünün yüzey antijeni epitoplarından 95-109 amino asit dizisinin Poliakrilik asid (PAA) ve poli (Vinil pirolidon-co-akrilik asit) (VPAA) kopolimeri gibi farklı polielektrolit ile fonksiyonel özellikteki biyokonjugatlarının geliştirilmesi sonucu antijenik peptidin immunojenliğinin arttırılması hedeflendiğinden, bu konjugasyon reaksiyonları sonucunda elde edilen biyokonjugatların yeni nesil aşıların geliştirilmesinde model olarak kullanılması amaçlanmıştır.

Yöntemler: Biyokonjugatlar, suda çözünebilir bir karboodiimid olan 1-etil-3-(3-dimetilaminopropil) karbodiimid yardımı ile, farklı karboksil aktivasyon mekanizmaları kullanılarak yapılmıştır. En iyi bağlanma metodu belirlendikten sonra, iki farklı polielektrolit ve farklı başlangıç mol oranları kullanılarak, bu yöntemin etkinliği gösterilmiştir. Ayrıca sentezlenen biyokonjugatlar, Floresans Spektrofotometre ve Boyut Eleme Kromatografisi gibi yöntemler kullanılarak analiz edilmiştir.

Bulgular: Kromatografik analiz sonuçaları polielektrolitler ile peptid molekülü arasında karbodiimid kullanılarak yapılan reaksiyonlarda, en iyi bağlanma metodunun Method 3 olduğunu göstermiştir. Bu metod kullanılarak yapılan biyokonjugasyon reaksiyonlarında peptid molekülünün konsantrasyonu arttıkça, peptidin polielektrolit ile bağlandığı ve oluşan biyokonjugatın molekül ağırlığının arttığı kromatografik yöntemler ile gösterilmiştir.

Sonuç: Seçilen aktivasyon yöntemi ile, polimer ve peptid molekülü arasında güçlü kovalent bağ oluşmasını sağlandığından, bu yaklaşımın geliştirilmesi antijenik peptidlerin polimerik taşıyıcılara bağlanmasında, yeni aşılar, tanı kitleri, farmasötik ve biyoteknolojik preparatların hazırlanması gibi alanlarda kullanılabilir.

Anahtar Kelimeler: biyokonjugasyon, sentetik peptidler, polielektrolitler, suda çözünebilen karbodiimid

Yazarlar arasında herhangi bir çıkar çatışması bulunmamaktadır.

Introduction

Hepatitis B, often referred to as silent killer, is one of the most important infectious diseases in the world. Interferon and various antiviral drugs have shown efficacy in inhibiting the replication of the Hepatitis B Virus, but the emergence of drug-resistant viral strains and failure in clearance of the virus are unsolved problems [1-3].

Peptides of various size and derivation are already widely used as therapeutic drugs [4]. However, in order to elicit maximum immunogenic response from synthetic peptide antigens, it is generally necessary to bind the peptide to a carrier molecule such as protein or polymer [5]. Most proteins are immunogenic to some extent and, finally, they are rapidly excreted by kidney ultrafiltration. Covalent polymer conjugation on the protein surface reduced or eliminated these problems, since the bound polymer behaves like a shield hindering the approach of proteolytic enzymes, antibodies, or antigen processing cell. Furthermore, the increase in the molecular weight of the conjugate helps to overcome the kidney elimination threshold [6]. In the treatment of cancer, a further advantage of peptides over larger proteins, such as full-length antibodies, is their superior ability to penetrate tumors [4, 7-8].

Polymer conjugation is of increasing interest in pharmaceutical chemistry for delivering drugs of simple structure or complex compounds such peptides, enzymes, and oligonucleotides [6]. Synthetic polymers may be conjugated covalently to a variety of natural or synthetic biomolecules for many diverse uses [9]. Polymeric systems can deliver drugs directly to the intended site of action, and can also improve efficacy while minimizing unwanted side effects elsewhere in the body [10].

Synthetic Polyelectrolytes (PEs) have been widely used to modify proteins via covalent attachment, increasing or reducing the immunoreactivity, and/or immunogenecity of originally antigenic proteins and improving their in vivo stability with prolonged clearance times. Besides, the conjugates of PE with individual microbe antigens develop strong protective properties and they can be considered as a new generation of vaccinating compounds [11]. It provides effective immune protection without traditional classical adjuvants [5].

The stability of the drug–polymer bond is crucial for polymer–protein conjugates and polymer–drug conjugates [12]. In principle, a number of non-covalent forces can contribute to the complex formation between polymers and proteins. It is reasonably certain that the interaction between proteins and polyvinylpyrrolidone (PVP) or other hydrophilic nonionic polymers and proteins involves the former as hydrogen bond acceptors [13-14].

The distribution of conjugates between polymeric carriers and drugs can be influenced by the properties of the polymer. Polyvinylpyrrolidone (PVP) can be linked with various comonomers in order to control the physicochemical properties of polymer carriers. Nonionic polymers do not interact with endothelial cells, but the increase in interaction between polymers and endothelial cells is parallel to the amount of charge or hydrophobic groups. The hydrophilic–hydrophobic balance of polymers is very important in bioconjugation with therapeutic drugs [15].

In this study, we established non-viral synthetic peptide conjugates using various coupling reactions. We have also developed new approaches to get these peptide bioconjugates: Different synthetic polianions, which are Polyacrylic acid (PAA) and Poly(vinyl pyrrolidone-co-acrylic acid) (VPAA) copolymer in 25:75 monomer composition, were used as a carrier molecule for the conjugation with the Hepatitis B Surface Antigen (HbsAg) peptide molecule. This peptide has Trp amino acid in N-terminal to make the peptide molecule florescent, and the HbsAg peptide is highly hydrophobic and contains one free amino group to bind the polyanions.

The interaction between antigenic HbsAg peptide and PE will be discussed on the basis of the experimental results obtained using a fluorescence spectroscopy and a size-exclusion chromatography (SEC) with on-line quadruple detection system: UV absorption (UV) and Right Angle Light Scattering (LS) detectors and the binding of peptide to PE.

Materials and Methods

The peptide sequence 95-109 of Hepatitis B Surface Antigen peptide was commercially purchased from BioBasic Inc. (Canada). The peptide sequence was: Trp-Leu-Val-Leu-Leu-Asp-Tyr-Gln-Gly-Met-Leu-Pro-Val-Cys-Pro-Leu. The molecular weight of the peptide was 1860 Da. The isoelectric point (pI) of the peptide was 3.1. Peptide was dissolved in 2% (v/v) DMSO-water, because it was hydrophobic and insoluble in aqueous medium.

PAA was from Aldrich (Germany) 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) was from Sigma Chemical Company (St. Louis, MO). Poly (N-vinyl pyrrolidone-2-co-acrylic acid) with VP/AA (25:75) monomer composition was synthesized as explained in literature [16]. NaH_2PO_4, $Na_2HPO_4.7H_2O$, NaCl, NaOH were obtained from Fluka, and NaN_3 was from Applichem. Ultra pure water was used in preparing solutions and was obtained from Millipore MilliQ Gradient system.

Preparation of the Peptide-Polymer Bioconjugates

All the bioconjugates between the HbsAg peptide and PE were prepared in aqueous solutions, and conjugation reactions were performed at pH 5 and pH 7, under ambient temperature, in a short time to avoid denaturation of the peptide.

First of all, we searched which EDC activation mechanism should be used during the conjugation reactions. To understand this, we tried 3 different ways to bind the

peptide molecule to PE and made a decision as described below. To understand and compare the features of synthesized bioconjugates, we used 2 different PEs; Polyacrylic acid (PAA) and copolymers of acrylic acid (AA) with vinyl pyrrolidone (VP) (VPAA). The concentrations of the said PEs were kept constant and were 0.5 mg/mL in all conjugation procedures, while the peptide concentrations were changed. The initial molar ratios of bioconjugates ($n_{Peptide}/n_{PE}$=1, 3, 5, 7, 9) were calculated using this equation:

$$n = c.N_A/M$$

M is the molecular weight of the components, N_A is Avagadro's Number, and c represents the concentration of the components in mg/mL.

Conjugation Reactions by Using Different PE Activation Mechanisms

In conjugation chemistry, amide bond formation is the reaction mostly used to introduce various linkers or spacers to biomolecules to be coupled, especially in the case of proteins [16]. Carbodiimides (CDI) are classified as zero-length cross-linking agents used to mediate the formation of amide linkages between carboxyl and amine groups [18-19]. 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) is a water-soluble derivative of CDI, and is widely used in the synthesis of peptides and hapten–protein and protein–protein conjugates [20]. The advantage of using these so called zero length cross-linking agents is that during cross-linking no additional chemical entities are introduced between the conjugating molecules [18-19, 21].

It is a well known fact that during EDC mediated coupling reactions of polymer, carboxyl groups are activated and form amide linkages with amine groups of peptide or proteins. Cross-linking of polymers with EDC involves the activation of carboxylic acid groups of polymer chains to give an O-acylisourea group. Cross-links are formed after a reaction with free amino groups (nucleophilic substitution) of the peptide chain, resulting in intermolecular cross-linking, respectively (Fig. 1).

Activation of carboxyl groups of PE was performed in water (pH 5.0) in a molar ratio 2 : 1 (EDC : PE). Generally, EDC is the most efficient at pH 4.5-7.2 because all reactions were carried out within this pH range.

In this study, activation of carboxyl groups in PE was performed using different types of activation procedures:

i. In the first activation method (*Method 1*) the polymer was activated using EDC at pH 5 and stirred 2 hr. After 2 hr, the mixture was adjusted to pH 7 with 1N NaOH, and the peptide was added. Stirring was allowed overnight.

ii. In the other procedure (*Method 2*) the polymer was activated using EDC at pH 5 and after that the peptide was added to the activated polymer solution, and stirring was allowed overnight.

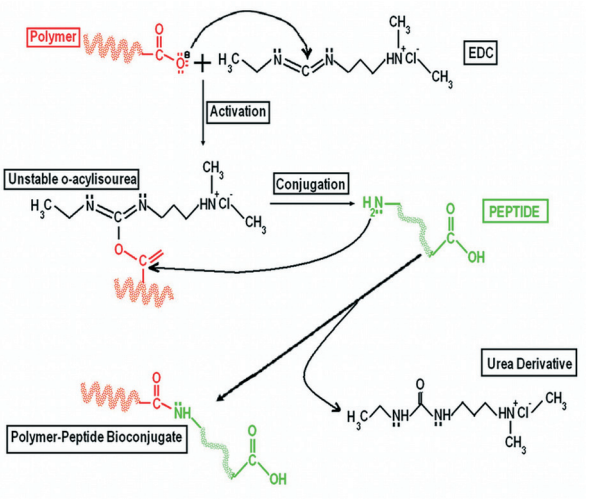

Figure 1. The activation carboxyl group of polymers, and coupling with the peptide to obtain a polymer-peptide conjugate [31].

iii. In the last activation reactions (*Method 3*), the polymer (PAA) and peptide were mixed at pH 5 and after stirring for 2 hr at room temperature, EDC was added into this mixture, and stirring was allowed overnight.

All the bioconjugates were purified using dialysis, and the purified conjugates were analyzed using a Fluorescence Spectrometer and a SEC to choose the best coupling procedure.

Conjugation Reactions Using Different Polyelectrolytes and Initial Molar Ratios

After choosing the best peptide coupling methods, we performed a wide range of conjugation reactions between the HBsAg peptide and 2 different PEs to prove the efficiency of *Method 3*.

PAA and VPAA were used as a synthetic carrier for the conjugation with the HBsAg peptide. The molecular weight of PEs used for the conjugation was:

1. Polyacrylic acid (PAA, Mw= 100.000)

2. Poly vinylpyrrolidone-co-acrylic acid copolymer (25:75, Mw= 80.000)

All the Peptide-PE conjugates were synthesized using carbodiimide condensation following modification procedures described early. The obtained bioconjugates were purified and then characterized using different spectrophotometric methods, such as a SEC and a Fluorescence Spectrophotometer.

Characterization of Synthetic Peptide-PE Conjugates

Fluorescence Measurements

Fluorescence emission spectra were obtained using a QM-4/2003 Quanta Master Steady State Spectrofluori-

meter (Photon Technology International, Canada) operating in quanta counting mode. The slits of excitation and emission monochromators were adjusted to 2 or 3 nm. The excitation was obtained at 280 nm for conjugates. Interaction with other macromolecules may result in changes in protein fluorescence spectra, which was characterized by the wavelength at the maximum emission (λ_{max}) and maximum fluorescence intensity (I_{max}).

Size-Exclusion Chromatography (SEC) Measurements

A Viscotek TDA 302 detector system with a refractive index (660 nm), a right angle light scattering (670 nm), and four-capillary differential viscometer detectors were used for on-line SEC signal detection. A separate UV detector, obtained from Viscotek, was connected to this detector system, and the detectors were in the following order: UV–LS–RI–VIS. 0.2 µm nylon pre-filter was used between the column and detectors. A HPLC pump, degasser, and auto sampler with 100 µL injection loop were built-in to the Viscotek GPCmax VE 2001 pump system, which was connected to the detectors. OmniSEC 4.1 software was used for the acquisition and analysis of SEC data.

Viscotek quadruple detector array was calibrated using a BSA monomer peak in the mobile phase of PBS with a flow rate of 1.0 ml/min. 0.185 and 0.66 [22,30] were the dn/dc value and extinction coefficient of BSA, respectively. Shim-Pack Diol 300 column with a length of 500 mm and an inlet diameter of 7.9 mm was used to separate the HBsAg peptide, PE, and peptide-PE conjugates. Elution was isocratic and at a flow rate of 1.0 ml/min. Phosphate-buffered saline (PBS) was prepared using ultra pure water from a Millipore MilliQ Gradient system, and consisted of 50 mM phosphate and 150 mM sodium chloride, with a pH of 6.0 and 7.0. Acetate buffer with 0.01 M CH_3COOH and 150 mM NaCl was prepared with ultra pure water, and adjusted to pH 5.0 with 1 M NaOH. 0.05% NaN_3 was added to the mobile phase solutions to prevent biological degradation of the columns. Buffer solutions were filtered through 0.45 µm Millipore cellulose nitrate filter, and were degassed before use.

Results

Fluorescence Measurements

The conjugation level of the HbsAg Peptide and different PEs were investigated depending on the PE types, activation methods of PEs, and molar ratios of components ($n_{Peptide}/n_{PE}$) for each bioconjugates. Fig.2. illustrate the fluorescence spectrum of the bioconjugates that synthesized 3 different activation mechanisms, and the HbsAg peptide molecule alone. We have modified and improved the coupling reactions using EDC in 3 different ways.

At pH 7, the wavelength (λ_{max}) of the HbsAg peptide was

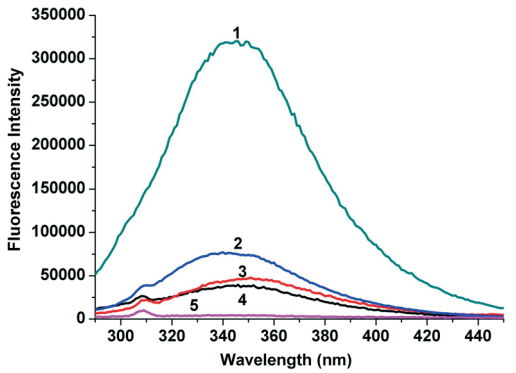

Figure 2. Fluorescence spectra of the conjugates at ratios of $n_{Peptide}/n_{PAA}$ that synthesized using 3 different activation methods at pH 7. (1) The peptide molecule alone λ_{max}=345 nm; (2) Method 3 λ_{max}=340 nm; (3) Method 2 λ_{max}=351 nm; (4) Method 1 λ_{max}=346 nm and (5) PAA alone.

345 nm. When the peptide was linked to PE molecules, there were some changes in the λ_{max} and (fluorescence intensity) I_{max} depending on the methods. Compared to the other methods, *Method 3* shows a near 5 nm decrease in λ_{max} and a noticeable change in I_{max}. It is a well-known fact that the tryptophan (Trp) fluorescence of proteins varies with conformational changes, resulting in a change in fluorescence parameters, such as emission maximum (λ_{max}), quantum yield, lifetime, and others [23,26-28]. This blue shift in the fluorescence spectrum of *Method 3* proves the covalent binding between the PE and HbsAg peptide molecule. As a result we subsequently used *Method 3* as the covalent conjugation mechanism in all experimental studies.

To investigate binding and structural properties of the PE-peptide conjugates, we used different initial molar ratios ($n_{Peptide}/n_{Polymer}$) and two different PEs, with different monomer compositions: PAA and Poly vinylpyrrolidone-co-acrylic acid copolymer (25:75). Modification of PAA with VP monomers makes the final polymer (Poly vinylpyrrolidone-co-acrylic acid) more non-ionic compared to PAA, and VPAA has a less functional group than PAA.

Proteins contain three amino acid residues that contribute to their ultraviolet fluorescence: tyrosine (Tyr-Y), tryptophan (Trp-W), and phenylalanine (Phe-F). Several useful reviews and monographs summarize their spectral properties. The maximum emission of tryptophan in water occurs near 350 nm and is highly dependent on polarity and/or the local environment [23-25, 28, 29, 32]. Tryptophanyls in proteins are sensitive to general solvent effects and also display a substantial spectral shift upon the formation of a hydrogen bond to the amino nitrogen, which can be regarded as a specific solvent effect. As a result, the emission of each tryptophan residue in a protein depends on the details of its surrounding environment [23, 32].

A schematic representation of the fluorescence results of Peptide-Poly vinylpyrrolidone-co-acrylic acid copolymer conjugates at different $n_{Peptide}/n_{VPAA}$ ratios at pH 7 is illustrated in Fig.3. When the peptide molecules increase within the conjugates, the I_{max} values increase proportionally. However, when we compare the results in terms of I_{max} of the peptide molecule alone, all the I_{max} for VPAA-Peptide conjugates decrease significantly. Also, there was a 5 nm blue shift illustrated in Fig.3b. This may be caused by the polymer molecules surrounding the peptide molecule in the aqueous solution. Generally, this is a result quenching as the tryptophanyl residue of the peptide is isolated from water.

As we can see from Fig. 4a, the only significant decrease is observed in the I_{max} of HBsAg Peptide-PAA conjugates in comparison to the pure HBsAg Peptide due to similar reasons

SEC Measurements

Similar to fluorescence results, the highest peak for the UV and Light Scattering (LS) measurements among 3 different EDC activation mechanisms was obtained for *Method 3*. Not only the intensity of the UV peak for *Method 3,* but also LS results show us that the binding level of the peptide molecule with the PE and the molecular size of resultant bioconjugates are maximum in *Method 3,* as *Method 3* has the highest peak in both the UV and LS chromatograms. It is thought that more conjugates are formed. The peak intensity is also the biggest as illustrated in Fig 5.

The UV chromatogram and Light Scattering (LS) graphics of VPAA- Peptide conjugates are illustrated in Fig. 6a and Fig. 6b. When the $n_{Peptide}/n_{VPAA}$ of conjugates increases so does the UV peaks of the bioconjugates . Changing the additional peptide amount of conjugates by changing $n_{peptide}/n_{PE}$, proves that each peptide to join the bioconjugate structures has a tendency to bind the VPAA copolymer. As seen in the results there are no free peptide peaks on the UV chromatograms. Only the ratio of $n_{Peptide}/n_{VPAA}$=1 has extra peaks in its chromatogram; Topuzogullari et al. previously described that this peak determines the retention time of PAA, which is 11.07 [30,31]. The high peak illustrated in Fig.6a, is retention volume at about 11, at a ratio of n=1, which can be described as PAA.

Similar to Fig. 5a and Fig. 5b, Fig.6b. also illustrates the LS graphs of the conjugates with different *n* values. The molecular weight of the conjugates increases depending

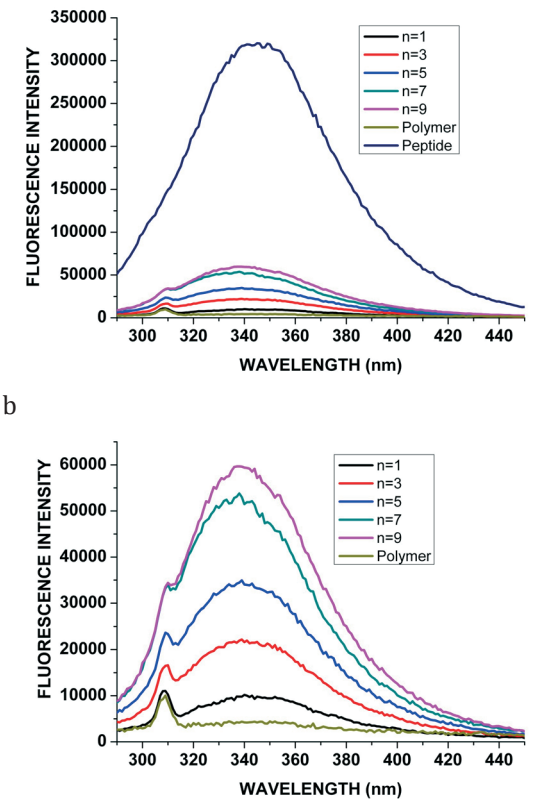

Figure 3. Fluorescence spectra of the conjugates of Polyvinyl pyrrolidone-co-acrylic acid (25:75) copolymer with Hepatitis B Surface Antigen Peptide (95-109) at different initial ratios, $n_{Peptide}/n_{VPAA}$: 1 (λ_{max}=340 nm), 3 (λ_{max}=340 nm), 5 (λ_{max}=339 nm), 7 (λ_{max}=340 nm) , 9 (λ_{max}=340 nm) at pH 7 (a) with pure HBsAg Peptide (λ_{max}=345 nm) (b) without pure HBsAg Peptide fluorescence spectrum.

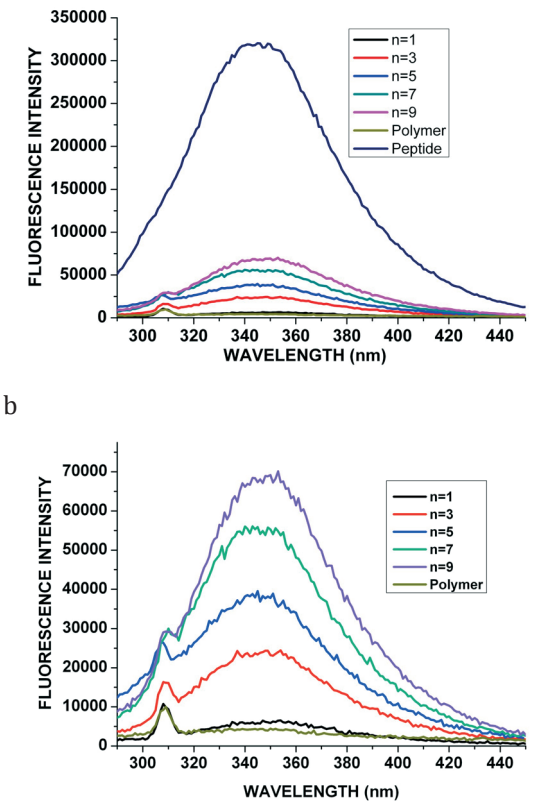

Figure 4.. Fluorescence spectra of the conjugates of PAA with HBsAg Peptide (95-109) at different initial ratios, $n_{Peptide}/n_{PAA}$: 1 (**λ_{max}**=345 nm), 3 (**λ_{max}**=345 nm), 5 (**λ_{max}**=345 nm) , 7 (**λ_{max}**=345 nm) , 9 (**λ_{max}**=345 nm) **(a)** with pure HBsAg Peptide (λ_{max}=345 nm) (**b**) without pure HBsAg Peptide fluorescence spectra.

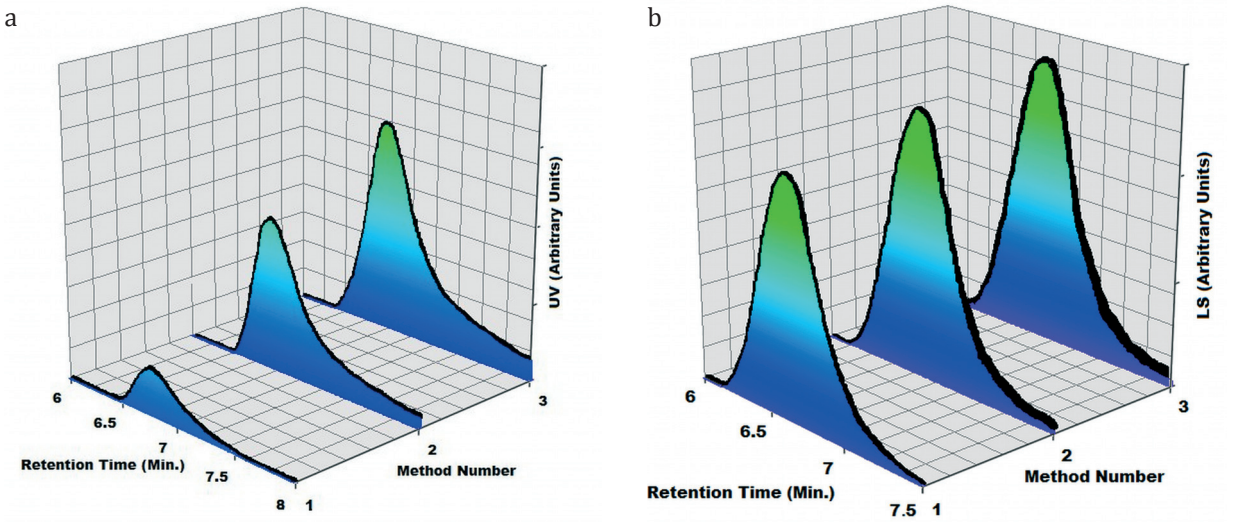

Figure 5. SEC chromatograms of the conjugates of PAA with HBsAg Peptide are synthesized using 3 different EDC activation methods at the same molar ratio and pH. (**1**) Method 1, (**2**) Method 2, (**3**) Method 3. (**a**) UV (**b**) Light Scattering (LS)

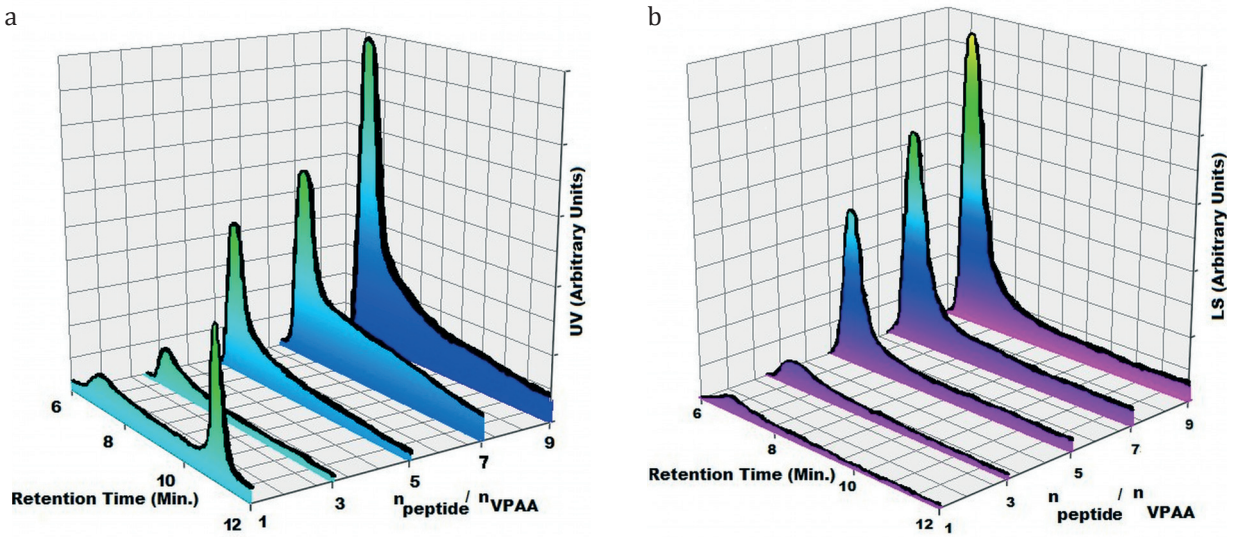

Figure 6. SEC chromatograms of the conjugates of VP/AA (25:75) copolymer with the HbsAg Peptide at different molar ratios ($n_{Peptide}$/ n_{VPAA}=1,3,5,7,9) at pH 7. **(a)** UV, (**b**) Light Scattering

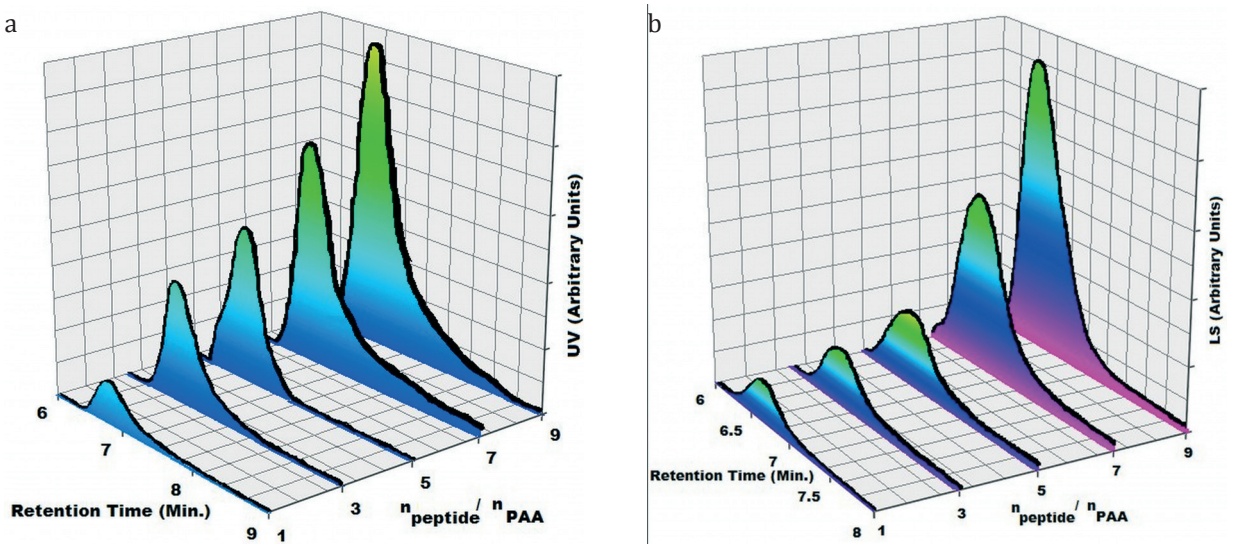

Figure 7. SEC chromatograms of the conjugates of PAA with Hepatitis B Surface Antigen Peptide (region 95-109) at different molar ratios ($n_{Peptide}$/n_{PAA}= 1, 3, 5, 7, 9) at pH 7. **(a)** UV, (**b**) Light Scattering

on molecule sizes. The more peptides bind to the polymer molecule, the higher the peaks. At the same time, as a general rule, the polymer or conjugates with a high molecular weight leave the column first; which is openly seen in the chromatograms.

When we take a look at the chromatograms given in Fig. 7, the results (shown in Fig. 6) are the same as the Peptide-VPAA conjugates. In other words, depending on the $n_{Peptide}/n_{PAA}$ (concentrations) of the Peptide-PAA conjugates there is a significant increase in both the UV absorbance and the LS spectrum.

Conclusion

The bioconjugation of peptides with polymers is a very important research field of biochemistry, bioengineering, and medicinal applications. The conjugates of such nonimmunogenic synthetic polyelectrolytes (PE) with microbial and viral protein and polysaccharide antigens given prior to inoculation confer protection against diseases. It provides effective immune protection without traditional classical adjuvants.

Two different polyelectrolytes and the HBsAg Peptide were covalently bound via carbodiimide activation of carboxyl groups of polyelectrolytes. The best carboxyl activation mechanism between the HBsAg and PEs was *Method 3,* which enabled an interaction between the polymer and peptide without any coupling reagents at first, and then allowed chemically coupling using EDC. It is obvious from the fluorescence and SEC measurements that binding is very strong with this method.

Besides, binding the highly hydrophobic HBsAg peptide molecule to PEs caused an increase in the solubility of the peptide molecule as all the resultant biocongates were entirely soluble in aqueous solution.

After choosing the coupling method, many conjugation reactions based on the various parameters, such as different PEs and $n_{Peptide}/n_{PE}$ of the conjugates etc., were performed to prove the efficiency of the coupling reactions, and as shown from the fluorescence spectrums and SEC chromatograms, when the concentration of peptide molecules to bind with the polymer increases, there is a significant increase in formation of bioconjugates; no remaining peptide molecules are monitored.

The development of these approaches to modify the immunogenecity of antigens will open perspectives for the creation of new vaccines, diagnostic, pharmaceutical, and biotechnological preparations.

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