Lysozyme Cross-Linked Antibodious Destroyers Against *Staphylococcus Aureus*

Staphylococcus Aureus'a Karşı Lizozim Çapraz Bağlı Antibadi Destroyerler

Research Article

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ABSTRACT

In the present study, we have provided a strategy to block staphylococcal protein A (SpA) which acts as a virulence factor and increase lysozyme activity against the staphylococcal infection. For this purpose, polymeric antibodious nano IgG has synthesized according to photosensitive micro emulsion technique and developed some binding parts on nano IgG for lysozyme. The bioconjugation method based on aminoacid monomer-protein cross-linking applying photosensitation and conjugation approach (ANADOLUCA) on micro and nano-structures by ruthenium-chelate based monomers has used. The antibacterial activities of polymeric nano-IgG and lysozyme cross-linked polymeric nano-IgG have investigated against to *Staphylococcus aureus*. The effects on *S. aureus* have determined using dilution plate method and turbidity method. In addition, the effects on *S. aureus* of lysozyme cross-linked polymeric nano-IgG have determined using confocal laser scanning microscopy. The biocompatibility of nanobioconjugates has determined by flow cytometry. The results have shown that nanobioconjugates could be used as an effective mimic and antimicrobial agents.

Key Words

Staphylococcus aureus, IgG, bioconjugation.

ÖZ

Bu çalışmada, stafilokokal enfeksiyonlara karşı lizozim aktivitesini arttıran ve virulans faktör gibi davranan stafilokokal protein A (SpA) bloklamak için bir strateji geliştirmiş bulunmaktayız. Bu amaç için, polimerik antibadimsi nano IgG destroyerler, fotosensitif mikro emülsiyon tekniğine göre sentezlenmiş ve nano IgG üzerinde lizozimler için bazı bağlanma bölgeleri geliştirilmiştir. Aminoasit-monomer çapraz bağlı fotosensitizasyon ve konjugasyon yaklaşımını (ANADOLUCA) mikro ve nano yapılara rutenyum-şelat tabanlı monomerlerle uygulama esasına dayanan biyokonjugasyon yöntemi kullanılmıştır. *Staphylococcus aureus*'a karşı polimerik nano IgG ve lizozim çapraz bağlı polimerik nano IgG'nin antibakteriyel etkileri araştırılmıştır. S. *aureus*'a etkiler dilüsyon pleyt yöntemi ve türbiditi yöntemine göre belirlenmiştir. Buna ek olarak, lizozim çapraz-bağlı nano-IgG'nin *S. aureus* 'a etkisi konfokal lazer taramalı mikroskop ile belirlenmiştir. Nanobiyokonjugatların biyo uyumluluğu akış sitometrisi ile belirlenmiştir. Sonuçlar, nanobiyokonjugatların etkili mimik ve antimikrobiyal ajanlar olarak kullanılabilirliğini göstermiştir.

Anahtar Kelimeler

Staphylococcus aureus, IgG, biyokonjugasyon.

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INTRODUCTION

The resistance of bacteria to antibiotics is causing scientists to search for alternative treatment methods for bacterial infections [1]. An antibiotic-free approach that uses antimicrobial enzymes conjugated to nanoparticles is a tremendous possibility to treat bacterial infection [2]. Also, biomolecules such as antibodies, antigen and peptides are used on the nanoscale because of their specific therapeutic and enzymatic activities against bacterial infection [3].

Staphylococcus aureus is an epidemiological bacterial pathogen whose leading significant diseases range from mild skin infection to deep systemic infections such as endocarditis, septic arthritis, osteomyelitis [4-7]. Staphylococcal protein A (SpA) is a major surface protein which exists in all strains of *S. aureus* [4]. SpA is a 42 kD bacterial surface protein with five homologous domains that have strong affinity for the Fc region of immunoglobulins [7-9]. Protein A interacts with immunoglobin (IgG) that (to) coat the surface of the cell during infection such that it camouflages the bacteria from being recognized by the immune system [7,10].

The treatment of Staphylococcal infections can be complicated because of the increase of multi-drug resistant *S. aureus* strains in hospitals and community environments [7,11]. Penicillin was used for the treatment of *S. aureus* infection in the early 1940s until it was reported that it was ineffective in 1945. Three years after starting to use methicillin in 1959, *S. aureus* isolates showed resistance to methicillin [5]. Currently, available treatments with broad-spectrum antibiotics are becoming less efficient [10]. The increase in both antibiotic-resistant microbes and health care costs are leading many scientists to attempt to develop cheaper and more efficient antimicrobial reagents [12].

Nanotechnology supplies a field of applied science and technology. Reducing the particle size of materials is an effective method to improve biocompatibility of nano-antibiotics. Nanoparticle-based antibiotics, therefore, are potential treatments for lethal bacterial infections. Using nanoparticles gives many advantages such as improved water solubility, stability, decreased toxicity to humans, or pharmacokinetics and distribution [11-13].

The objective of this study is to improve polymeric and antibodious nano IgG which has been synthesized according to a photosensitive micro emulsion technique developed for Staphylococcal protein A blocking. Nano IgG antibodious particles with photosensitive ruthenium hapten are also be useful in fluorescence detection and in tracking involves various bio-interactions. Photosensitive ruthenium based amino acid monomer hapten can be used in preparation of protein carriers through photosensitive polymerization and conjugation, and in detection and imaging of nanocarriers and its interaction [14]. After preparing antibodious nano-IgG particles, we have been developed some binding parts on nano IgG for lysozyme cross-linking to make lysozyme more active against the staphylococcal infection. Lysozyme is a ubiquitous enzyme in both prokaryotes and eukaryotes, which hydrolyzes β-1,4-glycosidic bonds between N-acetylglucosamine and N-acetylmuramic acid of the peptidoglycan layer in the bacterial cell wall, leading to bacterial lysis [8,15]. Lysozyme, however, shows no effective activity to oppose host molecules because of a modification of *S. aureus*` peptidoglycan which is occurred by O acetylation at the C-6 position of the N-acetyl muramic acid (NAM) [16,17]. Using bioconjugation and cross-linking method which is based on amino acid monomer-protein cross-linking using photosensitation and conjugation approach (ANADOLUCA) on micro and nano-structures by ruthenium-chelate based monomers has been helped us to avoid these problems.

Bioconjugate techniques provide opportunity for conjugating an antibody to another protein for several applications such as diagnostics, and therapeutics. Therefore, it is possible to design of reagent systems that can interact in high affinity with analyte. Using a number of conjugation and modification techniques, these specific antibodies can be modified to allow easy tracking in complex mixtures.

Due to using fluorescence featured chemical, the activity tests have carried out by fluorescence spectrophotometer. The particle size and zeta potential of both polymeric nano-IgG and lysozyme conjugated polymeric nano-IgG destroyers were analyzed by Zeta Sizer. Also, the protein concentration in the nanostructured IgG particles were determined by Bradford method. The antimicrobial activity of lysozyme cross-linked polymeric nano-IgG was investigated against *S. aureus* in vitro. The biocompatibility of the nanobioconjugates were determined by flow cytometry.

MATERIALS AND METHODS

Reagents

All the chemicals were analytical purity and were purchased from Sigma-Aldrich except ethylene glycol dimethacrylate (EDMA) purchased from Fluka A. G. (Buchs, Switzerland). All water used in the experiments was purified using a Barnstead (Dubuque, IA) ROpure LP® reverse osmosis unit and followed by a Barnstead D3804 NANOpure® organic/colloid removal and ion exchange packed bed system. The conductivity of water was at the level of 17 to 18 megohms-cm.

Cultures

Staphylococcus aureus (NRRL-B-767) were used as standard microorganisms in this study.

Synthesis of Polymeric Antibodious Nano-IgG Conjugates

Polymeric and antibodious nano-IgG particles were prepared by using photosensitive microemulsion polymerization technique. Microemulsion media was prepared dispersing 1.1 g polyvinyl alcohol (PVA) in 100 mL of deionized water. 1000 ppm nano-IgG solution was prepared with media pH: 7.0 phosphate buffer. Bis (2-2'-bipyridyl) Methacroyltyrosine-Methacroyltrosine-Ruthenium (II) (MATyr-Ru(bipyr)$_2$-MATyr) was synthesized as a functional monomer according to the previously published procedure [18]. 25 μL of MATyr-Ru(bipyr)$_2$-MATyr was added into 1000 ppm 500 μL of IgG solution and mixed for 4 h. The occurred IgG-MATyr-Ru(bipyr)$_2$-MATyr was added into dispersed PVA media. 0.02 g Ammonium per sulfate (APS) was dissolved in 45 mL of distilled water as an initiator and only 25 mL dispersed solution was added reaction media mixing for 48 h in daylight and at room temperature under N_2 atmosphere. IgG nanoparticles were separated from the reaction medium by centrifugation at 12000 rpm for 10 min and washed with phosphate buffer (pH 7.4) to remove unreacted soluble particles.

Analysing of Active Antigen Binding Sites of Nano IgG Particles

Active antigen binding sites of synthesized nano IgG particles was characterized by using the interactions between gold protein A and nano IgG with fluorescence spectrometer. After that, interactions between anti IgG antibody and nano IgG particles were investigated by using the same spectrophotometric method.

Gold-Protein A Interaction with Nano IgG for Staphylococcal Targeting

The characterization study was carried out for the understanding of active antigen binding sites of Nano IgG particles by using fluorescence spectroscopy. 500 μL of pH 7.4 phosphate buffer solution was added to IgG nanoparticles. Then, 5 μL of gold-protein A was added into 45 μL of IgG nanoparticles and the solution was mixed for a few minute. 500 μL of nano IgG in 2.5 mL of pH 7.4 phosphate buffer solution was excited at 290 nm, and emission was recorded at 581.02 nm. In the same way, 500 μL of gold-protein A conjugated nano IgG in 2.5 mL of pH 7.4 phosphate buffer solution was excited at 290 nm and emission was recorded at 581.02 nm.

Anti IgG Antibody Interaction with Nano IgG Particles Interacted Gold-Protein A

To prove active Fab region of nano IgG, anti IgG antibody was interacted with nano IgG conjugated gold-protein A. 0.3 mg anti IgG antibody was dissolved in 500 μL of pH:7.4 phosphate buffer solution. 50 μL of anti IgG antibody solution was added into the 50 μL of nano IgG conjugated gold-Protein A and the solution was mixed for a few minute. 500 μL of anti IgG antibody interaction with nano IgG conjugated gold-protein A in 2.5 mL of pH 7.4 phosphate buffer solution was excited at 290 nm, and emission was recorded at 340.00 and 581.02 nm.

Synthesis of Lysozyme Cross-linked Polymeric Nano-IgG Particles

250 μL of 0.1 M N-hydroxysuccinimide (NHS) and 250 μL of 0.4 M N-ethyl-N′-dimethylamino propyl-carbodiimide (EDC) were added into polymeric nano-IgG dispersion media prepared by dispering 1000 ppm nanoparticles in 1 μL of phosphate buffer (pH 7.4) and mixed for 2 h. Then, 500 μL of MATyr-Ru(bipyr)$_2$-MATyr was added into reaction medium and mixed for 24 h. 100 ppm lysozyme solution was added into the reaction media in company with 100 μL of APS then, mixed for 24 h. Lysozyme cross-linked nano-IgG was separated from the reaction solution by centrifugation at 12000 rpm for 10 min and washed with phosphate buffer (pH 7.4) to remove unreacted soluble particles.

Characterization Analysis

The particle size distrubition and zeta potential of both polymeric nano-IgG and lysozyme conjugated polymeric nano-IgG were analyzed by Zeta Sizer (Malvern Zeta Sizer Nano-ZS). The particle size of both polymeric nano-IgG and lysozyme conjugated polymeric nano-IgG were also determined by transmission electron microscopy (TEM).

Protein Determination in Nano IgG Proteinous Particles

Bradford Assay is a rapid and accurate method commonly used to determine the total protein concentration of a sample. The assay is based on the observation that the maximum absorbance for an acidic solution of Coomassie Brilliant Blue G–250 shifts from 465 nm to 595 nm when the protein binding occured. Both hydrophobic and ionic interactions stabilize the anionic form of the dye, causing a visible color change. Within the linear range of the assay (~5-25 mcg/mL), the more protein is present, the more Coomassie binds. It provides us a rapid and sensitive method for the quantitation of microgram quantities of protein [19]. The amount of proteins in the IgG nanostructures that were prepared by photosensitive microemulsion polymerization technique using photosensitive amino acid monomer and monomer hapten was determined by measuring the initial and final concentrations of IgG protein within the adsorption medium using Bradford Assay. A calibration curve was constructed with protein solution of known protein concentration (0.02–0.25 mg/mL) and used in the calculation of protein amount.

The Preparation of Microbial Suspension

Staphylococcus aureus (NRRL-B-767 isolate) which was conserved in a 15% glycerol media at -85ºC was inoculated nutrient agar surface and plates were incubated at 35-37ºC for 24 h for the activation. After 24 h, 11 mg *S. aureus* cell was scraped from the agar, and dissolved in 11 mL of nutrient broth to obtain 1 mg/mL cell stock.

Analysis of Antibacterial Activity

The lysozyme cross-linked polymeric nano-IgG (100, 200, 400 and 800 μL) and polymeric nano IgG (400 μL) were inoculated into sterile falcon culture tube containing 1.5 mL of nutrient broth with 1 mg/mL cell. 400 μL of polymeric nano IgG and lysozyme cross-linked polymeric nano-IgG were separately inoculated into 1.5 mL of nutrient broth medium as contamination control originating from lysozyme cross-linked polymeric nano-IgG. While *S. aureus* cell suspension was served as a positive growth control, the culture medium without a bacterium was used as a negative control.

For measurement of the antibacterial activity, cell suspension of *S. aureus* was threatened by polymeric nano IgG and lysozyme cross-linked polymeric nano-IgG in the shaking incubator for 24 h at 37ºC at 150 rpm. After this process, the cell suspension of *S. aureus* was count using dilution (1:9 dilution rates) plate method. In the method, decreasing of total cell number was determined counting of the colony number after incubation at 37ºC for 24 h. In addition to live cell counting, supernatant was taken and measured at 540 nm together with negative controls (blank) and positive controls. Decreasing of total cell mass was determined according to changing of turbidities.

Analysis of Biocompatibility

Cell Lines and Cell Culture

The Mouse embryonic fibroblast cell line (3T3) was used in these experiments. 3T3 cells were

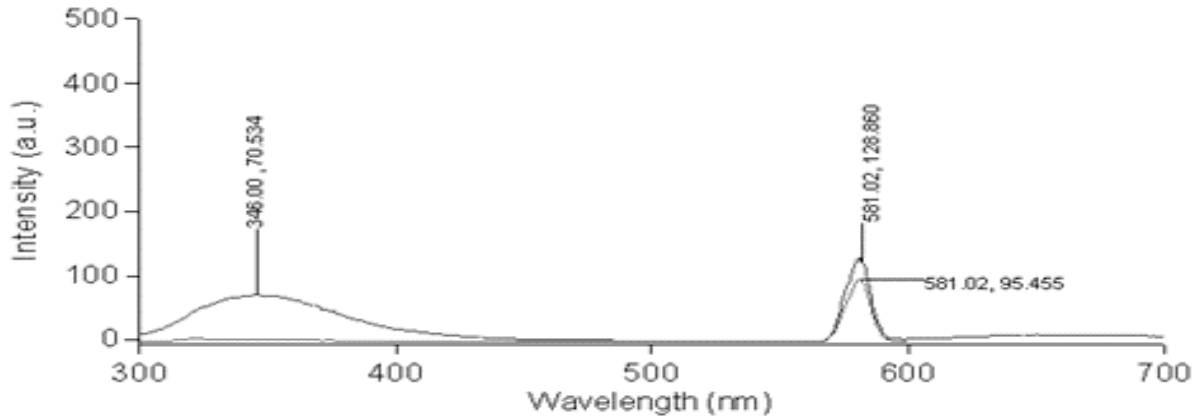

Figure 1. Gold-protein A interaction with nano IgG for staphylococcal targeting.

cultured for cell suspension at 1x10^6 cells/mL in Dulbecco's Modified Eagle's Medium (DMEM), containing with 10% (v/v) fetal calf serum and penicillin/streptomycin. The cell suspension was placed on a sterile plastic tissue culture plate and incubated at 37°C and 5% CO_2. Lysozyme cross-linked polymeric nano-IgG particles were interacted with 3T3 cells and then incubated in the cell culture for 24 h.

Flow Cytometry Analysis

3T3 cell solutions which have been incubated as explained above section were used to detect apoptosis and necrosis using the Annexin V-FITC Kit (BD Company, USA) according to the manufacturer's recommendations. Cell solutions were collected and harvested by centrifugation, washed twice in cold phosphate buffer saline (PBS) and resuspended in 100 mL of binding buffer. Then, 100 mL of each cell solutions (1x10^6 cells/mL) was taken and 5 μL of Annexin V- FITC and 5 μL of propidium iodide (PI) were added into the each samples and the cell solutions were incubated for staining for 15 min at room temperature (20–25°C) in the dark. Then, 400 μL of binding buffer was added into the each sample before analysing. The cells were counted with a flow cytometer (FACS Aria, BD Corporation, USA), FITC-conjugated Annexin V and PI emissions were detected using the Diva software and the percentage of cells undergoing apoptosis was determined by dual-color analysis. These stainings distinguish the cells into four subsets: viable cells (Q3) (no staining), early apoptotic cells (Q4) (annexin V positive and PI negative), late apoptotic cells (Q2) (annexin V positive and PI positive) and necrotic cells (Q1) (annexin V negative and PI positive). The experiments were repeated in triplicate.

RESULTS

Polymeric nano IgG particle synthesis was carried out by using photosensitive microemulsion polymerization technique and MATyr-Ru(bipyr)$_2$-MATrp which was used in synthesis leading to structure gaining fluorescence property. Analyzing of active antigen binding sites of nano IgG particle was carried out by using fluorescence spectrophotometer. Figure 1 shows quenching of IgG nanoparticles emission with the interaction of gold-protein A molecules. 500 μL of nano IgG particle in 2.5 mL of pH 7.4 phosphate buffer solution was excited at 290 nm, and emission was recorded at 581.02 nm. The fluorescence intensity value was measured as 95.455 (below peak). 500 μL of gold-protein A interacted nano IgG in 2,5 mL of pH 7.4 phosphate buffer solution was excited at 290 nm and emission was recorded at 346.00 and 581.02. The fluorescence intensity value was measured as 70.534 and 128.860 (above peak).

In this step, the multipoint interaction activity of nano IgG particle was proved by using fluorescence spectroscopy. Figure 2 shows fluorescence spectroscopy analysis of anti IgG antibody interaction with nano IgG conjugated gold-protein A emission. 500 μL of anti IgG antibody interaction with nano IgG conjugated gold-protein A in 2.5 mL of pH 7.4 phosphate buffer solution was excited at 290 nm, and emission was recorded at 340.00 and 581.02 nm.

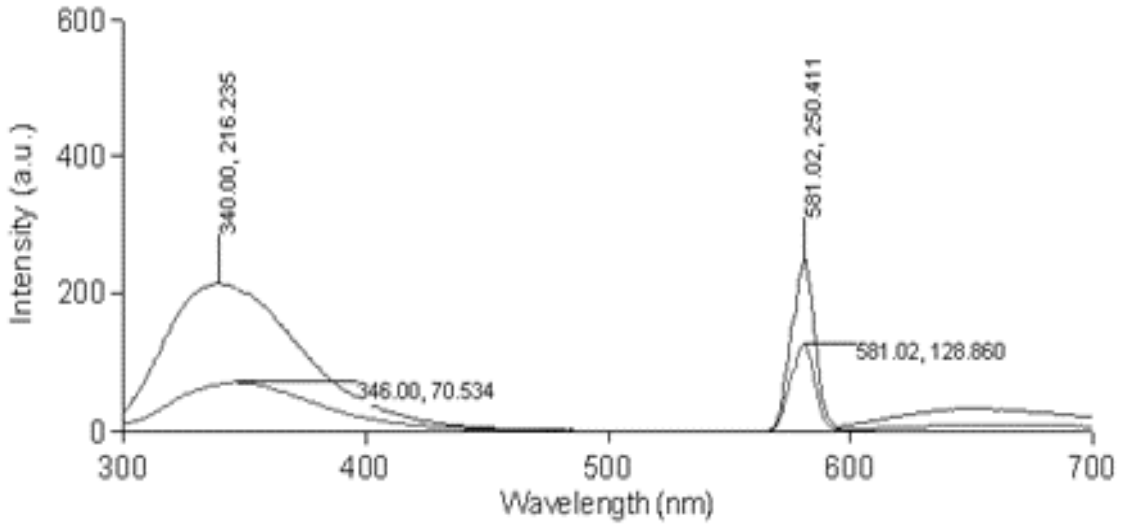

Figure 2. Anti IgG antibody interaction with nano IgG interacted Gold-protein A.

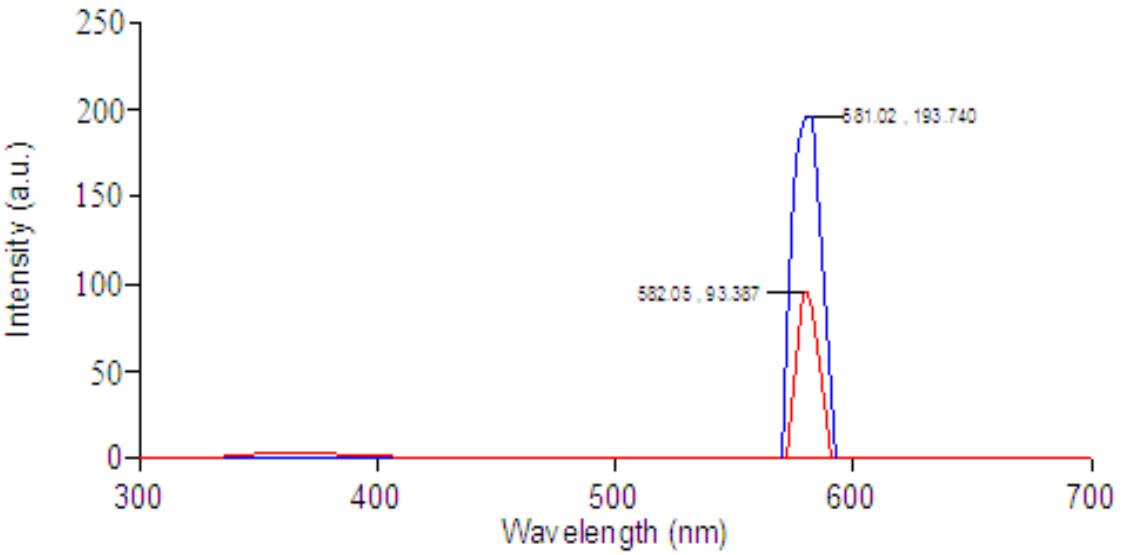

Figure 3. The spectrum of comparing polymeric nano-IgG and lysozyme cross-linked polymeric nano-IgG.

The fluorescence intensity value was measured as 216.235 and 250.411 (above peak) and the peak at 346.00 nm shows increasing and shifting to 340.00 nm. And peak at 581.02 nm shows increasing as a result of anti IgG interaction with nano IgG interacted gold-protein A.

Fluorescence spectrophotometer analysis was carried out to analyse lysozyme binding activity. Fluorescence spectrophotometer results showed that due to MATyr-Ru(bipyr)$_2$-MATrp mediating to bind lysozyme which is a molecule with 14 kDa to the structure, the fluorescence emission of polymeric nano-IgG has increased by becoming more stable (Figure 3).

The characterization studies have demonstrated that the average particle size of nano-IgG was 123.3 nm. After the conjugation of lysozyme, the average particle size (Figure 4) of lysozyme cross-linked polymeric nano-IgG was 285.8 nm. TEM images (Figure 5) showed that the

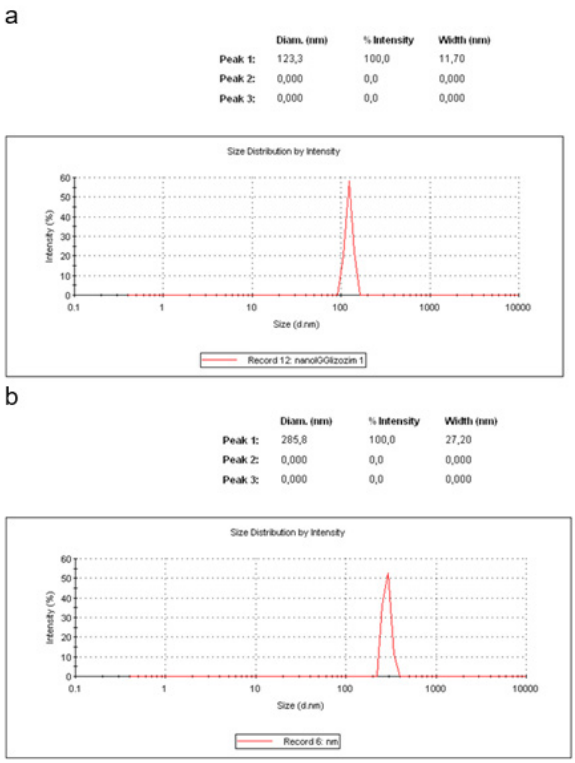

Figure 4. The average particle size of (a). nano-IgG, (b). lysozyme cross-linked polymeric nano-IgG .

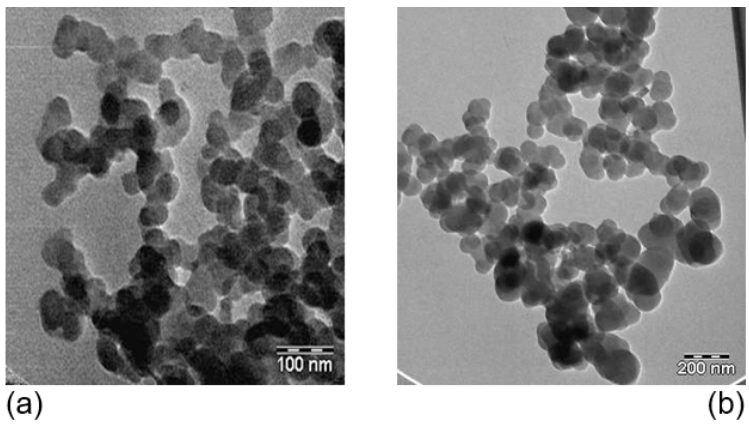

Figure 5. TEM image of nano (a). IgG particles, (b). IgG lysozyme bioconjugate particles.

nanoparticles size and size distribution too.

The zeta potential of polymeric nano-IgG was –22.1 mV. The isoelectric point (pI) value for lysozyme is 11.2 and the lysozyme molecules showed cationic feature at pH values below 11.2. Lysozyme, therefore, was cationic at pH 7.4. The new zeta potential value was recorded as –13.1 mV after conjugated of lysozyme. The cationic feature of lysozyme caused decrease in zeta potential which demonstrated that bioconjugation was carried out successfully.

Protein concentration in nano structured IgG was found to be 1.99 mg/mL according to Bradford assay while initial IgG concentration was 2.00 mg/mL. It showed that, there was no any loss in protein concentration in our nano structured protein synthesis method.

The activity of lysozyme cross-linked polymeric nano-IgG was analyzed in vitro. A significant increase in the death rate was

Table 1. The results of antibacterial activities of polymeric nano-IgG and lysozyme cross-linked polymeric nano-IgG destroyers.

	CFU/mL (Average)	Abs (540 nm)	The ratio of death (%)
Control	$160x10^7$	0.789	-------
400 μL of Polymeric Nano IgG	$154.5x10^7$	0.722	3.4
100 μL of Lysozyme Cross-linked Polymeric Nano-IgG	$151x10^7$	0.500	5.6
200 μL of Lysozyme Cross-linked Polymeric Nano-IgG	$138x10^7$	0.436	13.8
400 μL of Lysozyme Cross-linked Polymeric Nano-IgG	$100x10^7$	0.416	37.5
800 μL of Lysozyme Cross-linked Polymeric Nano-IgG	$89.8x10^7$	0.368	43.9

observed with increasing concentrations of nanobioconjugate. Treating suspension of bacteria with 800 μL of lysozyme conjugated polymeric nano-IgG, the death rate was increased to 43.8% (Table 1).

The flow cytometry analysis demonstrated that lysozyme cross-linked polymeric nano-IgG had no toxic effect on the mouse embryonic fibroblast cell although having a lethal activity against the bacteria suspension. Figure 7 showed necrotic cells ratio for 24 h incubation time (presented in the left-upper quadrant (Q1) was 2.0%, early apoptotic cells ratio (in the right-lower of the (Q4) and late apoptotic cells ratio (in the right-upper quadrant (Q2) were respectively 3.7% and 2.2%, respectively and viable cells ratio (in the left-lower quadrant (Q3) was 92.1%. Also, control cells (not interacted lysozyme cross-linked polymeric nano-IgG) were analysed by flow cyctometry and Q3 ratio (viable cells) was 97.8% .

DISCUSSION

The present study focused on blocking the staphylococcal protein A a wall-anchored protein with either four or five domains. Each domains bind to the Fc region of IgG. The result of the incorrect orientation between protein A and IgG, neutrophil Fc receptor cannot recognize antigen. The synthesis polymeric nano-IgG supplies us blocking the protein A. Preparing nanoparticles is a efficient method to synthesis homogeneous and stable nanoparticles. The nano size enabled to rapid and effective interaction with the staphylococcal protein A. Consequently, the interaction between protein A and nano-IgG decreases virulence of *S. aureus*.

In this study, we wanted to add a new ability to the polymeric nano IgG to increase antimicrobial activity. Although, lysozyme is an ubiquitous enzyme and part of the innate immune system, it does not effective against the *S. aureus* because of the different membrane structure. Using the technique of ANADOLUCA, a special binding site was prepared. Lysozyme, therefore, was cross-linked with polymeric nano-IgG. This appropriate binding sites allowed lysozyme to show activity. When lysozyme cross-linked polymeric nano-IgG was tested in *S. aureus*, the result demonstrated that the nanoparticles have an effective activity against the bacterial growth. In addition to this, it supplies us a non toxic health treatment method.

The nanobioconjugate which was synthesized with a method that is the first time in the literature offers many advantages. The microemulsion polymerization technique was used to synthesis of nanobioconjugates at daylight and room

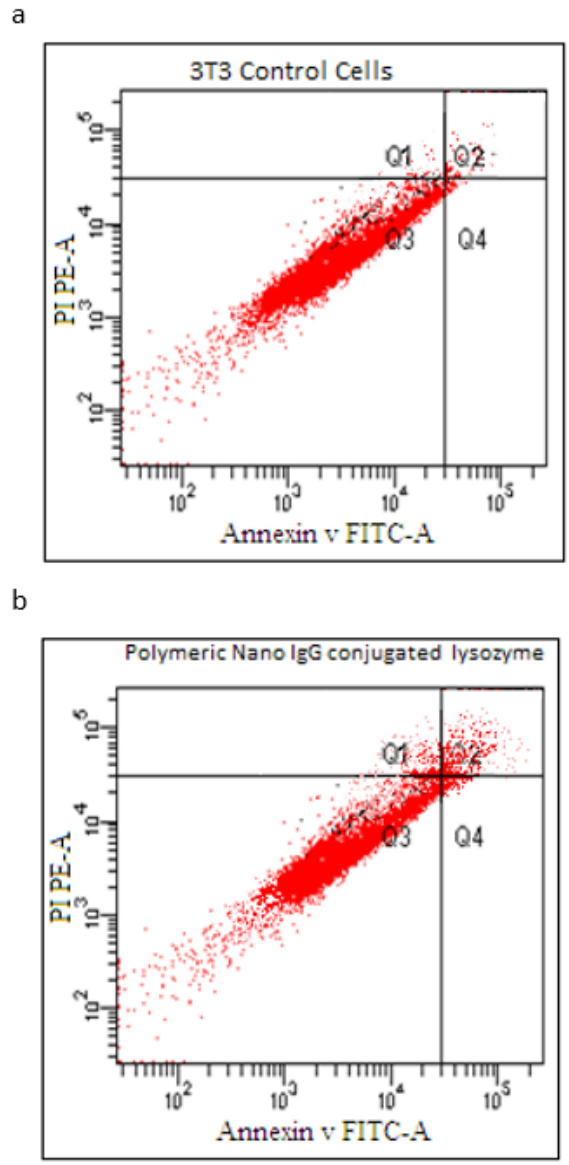

Figure 6. Flow cytometry data a) control cells, b) 3T3 cells were interacted with lysozyme cross-linked polymeric nano-IgG for 24 h. Early apoptotic cells are presented in the right-lower quadrant of the figure (Q4), late apoptotic cells in the right-upper quadrant (Q2), living cells in the left-lower quadrant (Q3), and necrotic cells in the left-upper quadrant (Q1).

temperature which causes long storage stability. Experiments demonstrated that their low amounts showed high activity. All these qualified facilities made them a cheaper treatment agent.

Due to ruthenium used synthesis stage, nanobioconjugate has had fluorescence feature to use development of biosensor for S. *aureus*. The biosensor which will be created this way, can be used to detect the presence of S. *aureus* if there is a risk of colonization of public places such as hospitals and/or nutriment. The determination and treatment will be made simultaneously thanks to the lysozyme conjugated structure.

After treating of cell suspension of *S. aureus* with polymeric nano IgG and lysozyme cross-linked polymeric nano-IgG, decreasing in the total cell number was determined by the average number of the colonies and turbidity (Table 1). The data in Table 1 showed that the *S. aureus* total cell number decreased from $160x10^7$ to $154.5x10^7$ after the interaction with the polymeric nano IgG and to $100x10^7$ after interaction with the lysozyme cross-linked polymeric nano-IgG. This showed that these materials showed antibacterial effects on *S. aureus*. Also, these values indicated that lysozyme cross-linked polymeric nano-IgG has more effective than polymeric nano IgG. As well as, these results showed that when the amount of the used lysozyme cross-linked polymeric nano-IgG has increased, the antibacterial effect has also increased. The Confocal Laser Scanning Microscopy results supported the antibacterial effect of lysozyme cross-linked polymeric nano-IgG (Figure 6). The images showed that the *S. aureus* total cell number decreased from 141 to 66 (53%) after interaction with 800 μL of lysozyme cross-linked polymeric nano-IgG for 24 h.

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Refefences

1. G.D. Wright, Bacterial resistance to antibiotics: enzymatic degradation and modification, Advanced Drug Delivery Reviews, 57 (2005) 1451-1470.
2. R. Satishkumar, A. Vertegel, Charge-directed targeting of antimicrobial protein-nanoparticle conjugates, Biotechnology and Bioengineering, 100 (2008) 403-412.
3. S.C. Yadav, A. Kumari, R. Yadav, Development of peptide and protein nanotherapeutics by nanoencapsulation and nanobioconjugation, Peptides, 32 (2011) 173-187.

4. E. Gustafssona, S. Karlssona, J. Oscarsson, P. Sogard, P. Nilsson, S. Arvidson, Mathematical modelling of the regulation of spa (protein A) transcription in *Staphylococcus aureus,* International Journal of Medical Microbiology 299, (2009) 65-74.
5. M. Askarian, A. Zeinalzadeh, A. Japoni, A. Alborzi, Z.A. Memish, Prevalence of nasal carriage of methicillin-resistant *Staphylococcus aureus* and its antibiotic susceptibility pattern in healthcare workers at Namazi Hospital, Shiraz, Iran International Journal of Infectious Diseases, 13 (2009) 241-247.
6. R. Saginur, K.N, Suh, *Staphylococcus aureus* bacteraemia of unknown primary source: Where do we stand? International Journal of Antimicrobial Agents, 32 (2008) 2-25.
7. N. Palmqvist, G.J. Silverman, E. Josefsson, A. Tarkowski, Bacterial cell wall-expressed protein A triggers supraclonal B-cell responses upon in vivo infection with *Staphylococcus aureus,* Microbes and Infection, 7 (2005) 1501-1511.
8. L. Pan, F. Yue, J. Miao, L. Zhang, J. Li, Molecular cloning and characterization of a novel c-type lysozyme gene in swimming crab Portunus trituberculatus, Fish & Shellfish Immunology, 29 (2010) 286-292.
9. M. Khalili, A. Liwo, H. A. Scheraga, Kinetic Studies of Folding of the B-domain of Staphylococcal Protein A with Molecular Dynamics and a United-residue (UNRES) Model of Polypeptide Chains, J Mol Biol., 355 (2006) 536-547.
10. T. J. Foster, Immune Evasion by Staphylococci, Nature Publishing Group 3, (2005) 948-958.
11. K. Greenhalgh, E. Turos, In vivo studies of polyacrylate nanoparticle emulsions for topical and systemic applications, Nanomedicine: Nanotechnology, Biology, and Medicine, 5 (2009) 46-54.
12. J.S. Kim, E. Kuk, K.N. Yu, J.H. Kim, S.J. Park, H.J. Lee, S.H. Kim, Y.K. Park, Y.H. Park, C.Y. Hwang, Y.K. Kim, Y. S.Lee, D.H. Jeong, M.H. Cho, Antimicrobial effects of silver nanoparticles, Nanomedicine: Nanotechnology, Biology, and Medicine, 3 (2007) 95-101.
13. A. Nanda, M. Saravanan, M. Phil, Biosynthesis of silver nanoparticles from *Staphylococcus aureus* and its antimicrobial activity against MRSA and MRSE, Nanomedicine: Nanotechnology, Biology, and Medicine, 5 (2009) 452-456.
14. R. Say, Photosensitive amino acid-monomer linkage and bioconjugation applications in life sciences and biotechnology. World Intellectual Property Organization Patent Scope. 2011,www.wipo.int/patentscope/search/en/WO2011070402 (Pub. No.:WO/2011/070402; Int. Appl. No.: PCT/IB2009.055707).
15. M. Liu, S. Zhang, Z. Liu, H. Li, A. Xu, Gene, 367 (2006) 110-117.
16. I. Fedtke, F. Gotz, A. Peschel, Bacterial evasion of innate host defenses–the *Staphylococcus aureus* lesson, International Journal of Medical Microbiology, 294 (2004) 89-194.
17. A. Bera, R. Biswas, S. Herbert, E. Kulauzovic, C. Weidenmaier, A. Peschel, F. Gotz, Influence of wall teichoic acid on lysozyme resistance in *Staphylococcus aureus,* Journal of Bacteriology, 189 (2007) 280-283.
18. R. Say, R. Keçili, Ö. Biçen, F. Yılmaz, D. Hür, A. Denizli, A. Ersöz, A novel nanoprotein particle synthesis: Nanolipase, Process Biochemistry, 46 (2011) 1688-1692.
19. M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Analytical Biochemistry, 72 (1976) 248-254.