Novel Protein Photocrosslinking and Cryopolymerization Method for Cryogel-Based Antibacterial Material Synthesis

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ABSTRACT: Lysozyme (Lys), also known as muramidase or *N*-acetylmuramide glycanhydrolase, is a family of enzymes that damage bacterial cell walls by catalyzing the hydrolysis of 1,4-β-linkages between *N*-acetylmuramic acid and *N*-acetyl-D-glucosamine residues in a peptidoglycan and between *N*-acetyl-D-glucosamine residues in chitodextrins. In this study, Lys was enhanced with a photosensitive facility by ruthenium chelate based monomers with a photosensitization and conjugation approach [amino acid (monomer) decorated and light underpinning conjugation approach]. Then, photosensitive Lys was allowed to interact with methacryloyl amido tyrosine (MATyr) monomer, which included tyrosine parts in its structure similar to those in the Lys structure. Two different types of cryogels including Lys–MATyr in their macropores were synthesized, Lys–MATyr cryogel and Lys–MATyr–clay cryogel. These two materials were used for the measurement of the antibacterial activity on Gram-positive and Gram-negative bacteria. The effects of the modified Lys on *Staphylococcus aureus* and *Micrococcus luteus* were determined with a dilution plate method. The effects on *Escherichia coli* and *S. aureus* were determined with a turbidity method. According to the data, the Lys–MATyr–clay modified embedded cryogel was effective on Gram-positive bacteria, and the Lys–MATyr modified embedded cryogel was so effective on Gram-negative bacteria. © 2011 Wiley Periodicals, Inc. J Appl Polym Sci 125: 145–151, 2012

Key words: biomaterials; conjugated polymers; enzymes

INTRODUCTION

With the growing public health awareness of disease transmission, cross-infection, and malodors caused by microorganisms, the use of antimicrobial materials has increased in many application areas, especially in protective clothing for medical and chemical workers and first receivers, sportswear, underwear, and other health-related products. Antimicrobial materials can be chemically engineered by the addition of functional antimicrobial agents (e.g., *N*-halamines) onto the surface or within the matrix to either kill or inhibit the growth of microorganisms to better protect the substrates from biological activities.[1] Peptidoglycan —the significant structure of species of Bacteria— can be destroyed by certain agents. One such agent is the enzyme lysozyme (Lys), a protein that breaks the β-1,4-glycosidic bonds between C-1 of *N*-acetylmuramic acid and the C-4 of *N*-acetylglucosamine of the peptidoglycan, thereby weakening the cell wall. Water then enters the cell, and the cell swells and eventually bursts, a process called *lysis*.[2,3] Because of its lysis-causing properties, Lys has important role in the development of antibacterial material studies. Besides its antibacterial activity, lysozyme has many other functions including inactivation of certain viruses,[4] surveillance of membranes of mammalian cells,[5] enhancing phagocytic activity of polymorphonuclear leukocytes[6] and macrophages,[7] stimulation of monocytes,[8] antitumor activity[9] and induction of fusion of phospholipids vesicles.[10] Lys is ubiquitously present in various human tissues and secretions, and it is also found in a variety of vertebrate cells and secretions, such as in the spleen, milk, tears, and egg whites.[11,12]

Cryogels that have interconnected macropores or supermacropores with a pore size range of 10–100 μm are gel matrices, which are formed in frozen solutions of monomeric or polymeric precursors. That continuous system of interconnected macropores is attractive in materials for chromatography and efficient carriers for the immobilization of imprinted materials, cells, or biomolecules.[13] This cryogel sponge not only has found application as an attractive stationary phase in bioseparation, but it also has potential application as a porous biocompatible matrix.[14] These materials can be prepared under solvent-freezing conditions and can be used in the field of biomaterials applications.[15–23] Cryogels are highly

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elastic, and about 70% of the total liquid inside can be removed by mechanical compression. Compressed cryogels swell again when they come in contact with liquid, and their original shape is restored in less than 1 min.[24]

The amino acid (monomer) decorated and light underpinning conjugation approach (ANADOLUCA) method provides a strategy for the preparation of photosensitive ruthenium (Ru)-based amino acid monomers and oligomers and amino acid monomer–protein crosslinking using photosensitization and conjugation approach (ANADOLUCA) on microstructures and nanostructures by Ru-chelate-based monomers. Indeed, the method provides efficient binding of proteins as well as many uses in the production of reusable enzymes, reusable separation solid phase systems based on affinity chromatography, theranostics, nanoprotein carrier, receptor targeted nanocargoes, manageable imaging and detection technologies.[25]

In this study, first, Lys was interacted with the Ru-based monomers methacryloyl amido tyrosine (MATyr)–Ru–MATyr to gain photosensitive features and was then interacted with MATyr monomer, which included tyrosine parts in its structure similar to those in the Lys structure. At the end of this interaction, the tyrosine residues of the MATyr monomer bound the tyrosine sequences of the Lys enzyme. This process caused an enhancement of the activity of Lys enzyme for damaging the bacterial cell wall. Second, an acrylamide (AAm)-based cryogel was prepared with AAm as the monomer with the crosslinker *N,N′*-methylene bisacrylamide (MBAAm); the reaction was induced by the redox initiator pair ammonium persulfate (APS) and *N,N,N′,N′*-tetramethylethylenediamine (TEMED). The prepared Lys–MATyr solution was added to the cryogel solution before the freezing step in the cryogelation process. After the freezing–thawing step, an AAm-based cryogel with Lys–MATyr in its macroporous structure was prepared. On the other hand, with the same amounts of monomer and crosslinking agent, another cryogel solution was prepared. After this cryogel solution was mixed with Lys–MATyr solution, organoclay was added. A cryogel containing both Lys–MATyr and clay in its macropores was synthesized at the end of the freezing–thawing step. So, a new antibacterial material with Lys–MATyr with a high surface area with clay and that was more active material against bacteria was created. *Staphylococcus aureus* and *Micrococcus luteus* were chosen as the Gram-positive bacteria, and *Escherichia coli* was chosen as the Gram-negative bacteria. The studies showed that our synthesized cryogel with Lys–MATyr in its pores was effective for both Gram-positive bacteria and Gram-negative bacteria as an antibacterial material.

EXPERIMENTAL

Materials

The functional monomer MATyr was synthesized according to a published procedure.[25] AAm (99%) was bought from Sigma-Aldrich. MBAAm was purchased from Fluka Chemica, and TEMED was bought from Fluka BioChemica. APS was obtained from local sources. Lys was bought from Sigma-Aldrich.

Cultures

Three kinds of different bacteria, including Gram-positive bacteria [*S. aureus* (NRRL-767) and *M. luteus* (NRRL-B-4275)] and Gram-negative bacteria [*E. coli* (ATCC 25922)], were used as standard microorganisms in this study.

Methods

Chemical synthesis of MATyr

Tyrosine amino acid was dissolved in a 1*M* aqueous solution of NaOH in a round-bottom flask. A solution of methacryloyl benzoriazole in 25 mL of 1,4-dioxane was slowly added to the amino acid solution. The reaction mixture was allowed to stir for 10–20 min at room temperature. The completion of the reaction was monitored by thin layer chromatography (TLC); after the reaction finished, 1,4-dioxane was evaporated *in vacuo*. The residue was diluted with water and extracted with ethyl acetate (3×50 mL) to remove 1*H*-benzotriazole. The collected water phases were neutralized to pH 6–7 with a 10% water solution of HCl (the pH needed to remain around 6–7 to prevent the possible polymerization of methacryloyl groups in acidic medium). Water was removed via a rotary evaporator to give MATyr in a 90% yield.[25]

Chemical synthesis of the photosensitive Ru-based amino acid–monomer bis(2-2′-bipyridyl) MATyr–MATyr–Ru(II) [MATyr–Ru–MATyr]

First, dichlorobis(2–2′-bipyridyl)ruthenium(II) [$RuCl_2$(bipyr)$_2$] was synthesized according to a previously published method:[26] $RuCl_2$(bipyr)$_2$ (0.1 g, 1 equiv) was dissolved in water, and the solution was cooled to 0°C. Then, ethylenetriamine and an aqueous solution of MATyr (0.1 g, 2 equiv) were added dropwise into that solution and stirred at room temperature for 30 min. The mixture was heated to 80°C and refluxed for about 24 h. The brown complex (MATyr–Ru–MATyr) was filtered off, washed with ether, and dried *in vacuo* (mp = >200°C).

Synthesis of the photosensitive poly[bis(2-2′-bipyridyl)–MATyr–MATyr–Ru(II)]

The photosensitive polymer could be prepared by a conventional polymerization reaction. First, the

necessary amount of bis(2-2′-bipyridyl)–MATyr–MATyr–Ru(II) monomer in dimethyl sulfoxide was mixed to obtain a photosensitive oligomer (dissolved in water). The polymerization reaction took place in the presence of 2-2′-azobisisobutyronitrile (AIBN) as an initiator. For the matrix-assisted laser desorption/ionization (MALDI)–time of flight (TOF) mass spectroscopy (MS) analysis, 2 μL of a $[Ru(bpy)_2MATyr–MATyr]_n$ solution was mixed with 23 μL of a 10 mg/mL solution of α-cyano-4-hydroxycinnamic acid in acetonitrile/0.3% trifluoracetic acid (TFA). The acceleration voltage was set to 20 kV, the delay time was 400 ns, the grid voltage was 70%, and the laser intensity was 2092 in reflector mode.[27]

Preparation of Lys–MATyr

To prepare the MATyr–Lys solution, first, 0.3 g of MATyr was dissolved in 5 mL of deionized water. In the other reaction, 0.3 g of Lys was dissolved in 5 mL of deionized water and interacted with 25 μL of MATyr–Ru–MATyr. After that, 3 mL was taken from each solution and mixed at 100 rpm for 3 h. This prepared MATyr–Lys solution was used in the cryogelation process to immobilize MATyr–Lys into cryogel macropores.

Preparation of the Lys–MATyr and Lys–MATyr–clay cryogels

The Lys–MATyr-doped AAm cryogel was prepared by the free-radical cryopolymerization of a monomer solution of AAm with the crosslinker MBAAm and initiated by TEMED and APS in a plastic Petri dish. As a general procedure for preparing a cryogel matrix, first, the AAm monomer and crosslinker MBAAm were dissolved in deionized water with stirring. The monomer concentration was 7% (w/v) with an AAm/MBAAm molar ratio of 10 :1. Second, the Lys–MATyr mixture (2% w/v of the total mass of AAm + MBAAm) was added to the solution of monomer and crosslinking agent (AAm + MBAAm) and mixed at 150 rpm for 30 min. Then, the prepared solution (AAM + MBAAm + Lys–MATyr) was cooled for 3–4 min in an ice bath. TEMED (1% w/v of the total mass of AAM + MBAAm + Lys–MATyr) and APS (1.2% w/v of the total mass of AAM + MBAAm + Lys–MATyr) were quickly added to the previous solution as redox initiators. The mixture was stirred for 1–2 min and was then poured into the Petri dish. This cryogel matrix in the Petri dish was frozen at −18°C for 16 h. Finally, it was thawed at room temperature. The formed cryogel material was washed with deionized water three times to remove unreacted soluble materials.

The Lys–MATyr–clay-doped AAm cryogel was prepared with the same cryogelation procedure. Organoclay (2% w/v of the total mass of AAm + MBAAm) was added to the cryogel solution and mixed at 150 rpm for 2 h before the redox initiator pairs were added. After the freeze-drying process, an AAm cryogel with Lys–MATyr–clay in its macropores was synthesized. This material was washed with deionized water three times.

Both of these cryogels were kept in 0.02% sodium azide solution until use.

Preparation of the microbial suspension

S. aureus and *M. luteus* were grown on nutrient agar plates at 37°C for 24 h. A 50-mg cell mass of these microorganisms was transferred to fresh falcon tubes by a loop. The suspensions of microorganisms were prepared with 100 mL of 0.1*M* phosphate buffer (pH = 6).

Characterization of the Lys–MATyr and Lys–MATyr–clay cryogels

The characterization of the cryogel bands was made by swelling tests, scanning electron microscopy (SEM) images, and Fourier transform infrared (FTIR) spectroscopy. The swelling ratios of the cryogels were determined in distilled water. Initially, the Lys–MATyr and Lys–MATyr–clay cryogel samples were washed until the washing was clear. Then, they were dried to a constant mass, and the mass of the dried sample was determined (±0.0001 g). These dried samples were placed into a swelling medium at room temperature for 4 h. Then, the mass of the wet sample was determined. The swelling ratio was calculated with Eq. (1):[28–32]

$$\text{Swelling ratio}(\%) = (W_s - W_0/W_0) \times 100 \qquad (1)$$

where W_0 and W_s are the weights of the cryogels before and after swelling, respectively.

The polyacrylamide-based cryogel was characterized by FTIR spectroscopy (PerkinElmer model 2000) in attenuated total reflection mode. The surface areas of the cryogel samples were determined by a surface adsorption analyzer (NOVA 2200 surface area and pore size analyzer, Quantachrome Instruments) according to the Brunauer–Emmett–Teller method. For this purpose, 1 g of cryogel sample was placed on the surface adsorption analyzer, and aqueous nitrogen was passed through the cryogel samples to remove water from the pores of cryogel. Then, analysis was started for 50 points of the sample according to the nitrogen adsorption principle. The cryogel samples were dried under a nitrogen atmosphere and then vacuum-dried overnight before they were gold-coated. The SEM images were taken by using SEM-LEO S440, Cambridge.

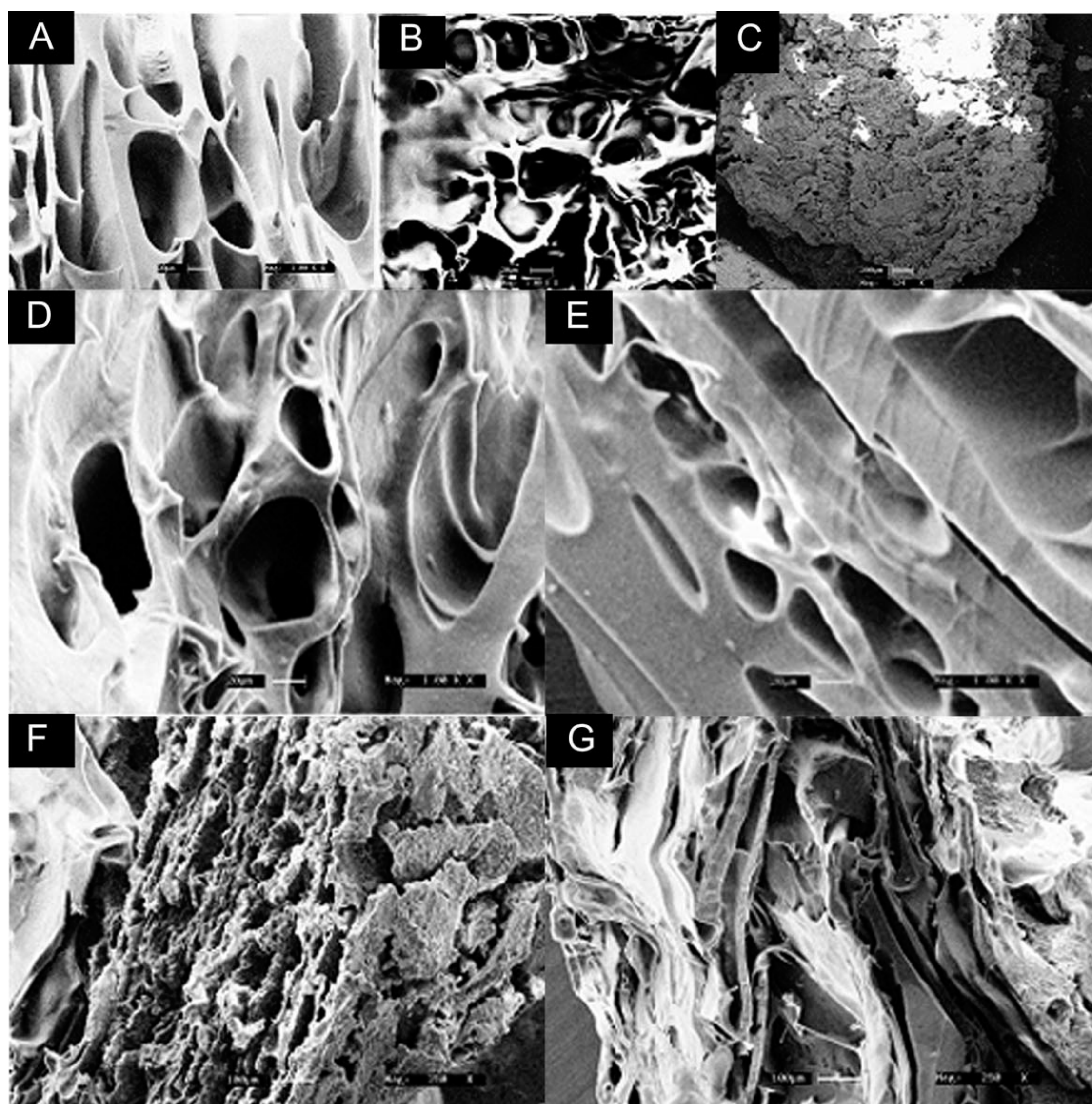

Figure 1 SEM images of (a) AAm cryogels (pore size = 20–40 μm), (b) Lys–MATyr–AAm cryogels (pore size = 50–60 μm), (c) Lys–MATyr–clay AAm cryogels (pore size = 50–70 μm), (d) Lys–MATyr–AAm cryogel that interacted with *M. luteus* (pore size = 50–60 μm), (e) Lys–MATyr–AAm cryogel that interacted with *S. aureus* (pore size = 50–70 μm), (f) Lys–MATyr–clay AAm cryogels interacted with *M. luteus* (pore size = 50–70 μm), and (g) Lys–MATyr–clay AAm cryogels interacted with *S. aureus* (pore size = 50–70 μm).

RESULTS AND DISCUSSION

Characterization of the antibacterial materials

The swelling ratios of the prepared cryogels were found to be 960, 940, and 992% for the AAm cryogel, Lys–MATyr cryogel, and Lys–MATyr–clay cryogel, respectively. According to these results, the swelling ratio of the nonembedded AAm cryogel was found to be 960%, and the swelling ratio of the Lys–MATyr–AAm cryogel was found to be 940%. When this swelling ratio value was compared to the swelling ratio value of the nonembedded AAm cryogel, it was seen that the hydrophilicity of the AAm cryogel decreased because Lys–MATyr was bound to the macropores of the cryogel. When the organoclay interacted with Lys–MATyr and was embedded in the macropores of the cryogel, the swelling ratio was found to be 992%. This higher swelling value meant that a higher water-capturing capacity was observed with the Lys–MATyr–clay cryogel, which had a high surface area because of the most porous structure of both the clay and cryogel. The swelling ratio values were the results of five replicates, and the standard deviation was about ±2,3%.

The morphology of these three types of cryogels was investigated by SEM (Fig. 1). The cryogel samples were dried under a nitrogen atmosphere and then vacuum-dried overnight before they were gold-coated. The SEM images were taken by with the SEM-LEO S440.

TABLE I
Total Colony Amount of Materials

Material	Total colony amount (cfu)
MATyr–Lys	15
MATyr–Lys–clay	1

The surface areas of the Lys–MATyr cryogel and Lys–MATyr–clay cryogel were found to be 19.8 and 25.1 m^2/g, respectively.

The polyacrylamide-based cryogel had a carbonyl band at 1648 cm^{-1} and an N—H bending band 1600 cm^{-1}, characteristics of the —$CONH_2$ group containing AAm; N—H stretching at 3185 cm^{-1}; and a C—H bending band at 1414 cm^{-1} according to the FTIR results.

Characterization of MATyr

^{1}H-NMR [500 MHz, deuterated dimethyl sulfoxide (DMSO-d_6), δ, ppm]: 7.35 (d, J = 8.05 Hz, 2H), 7.05 (d, J = 8.40 Hz, 2H), 6.29 (s, 1H), 5.90 (s, 1H), 3.15 (dd, J = 4.49, 14.25 Hz, 1H), 3.02 (dd, J = 4.02, 14,12 Hz, 1H), 2.90 (dd, J = 7.75, 14.20 Hz, 1H), 2.00 (s, 3H). ^{13}C-NMR (125 MHz, DMSO-d_6, δ, ppm): 170.9, 165.8, 149.6, 135.9, 130.9, 128.0, 121.8, 115.6, 55.9, 37.0, 18.5.

Characterization of bis(2-2′-bipyridyl) bis(MATyr)–Ru(II)

ANAL.. Calcd for $C_{46}H_{44}N_6O_8Ru$: C, 60.72%; H, 4.87%; N, 9.24%. Found: C, 61.54%; H, 4.63%, N, 10.56%. ^{1}H-NMR (500 MHz, CD_3OD, ppm): 9.78 (1H, s), 7.8 (1H, d, J = 8.15 Hz), 7.73 (t, 1H, J = 4.1 Hz), 7.64 (t, 2H, J = 4.11 Hz), 7.47–7.41 (p, 2H), 7.3–7.11 (m, 11H), 7.05 (t, 3H, J = 8.18 Hz), 6.95 (d, 2H, J = 8.18 Hz), 6.78 (s, 9H), 6.69 (d, 1H, J = 12.75 Hz), 6.34 (s, 1H), 5.84 (s, 1H), 4.5 (s, 1H), 4.25 (s, 2H), 2.06 (s, 3H), 1.91 (s, 3H).

According to MALDI–TOF–MS analysis; the ion peaks at 79, 128, and 155 m/z were related to bipyridyl. The 101 and 413 m/z peaks showed Ru and $Ru(bpy)_2$, respectively. The 250, 599, and 755 m/z data showed the MATyr monomer, Ru–$(MATyr)_2$, and Ru(bpy)–MATyr complex, respectively.

Antibacterial activity tests

For the determination of the microbial mass on the materials, the Lys–MATyr cryogel and Lys–MATyr–clay cryogel were inoculated on nutrient agar medium with the aim of determining their microbial masses. After incubation at 37°C for 24 h, the colonies were counted (Table I). The results show that these two types of cryogel materials had a few microbial contaminants, and the Lys–MATyr–clay cryogel had a lower microbial content than the Lys–MATyr cryogel.

For the measurement of antibacterial activity, cell suspensions of *S. aureus* and *M. luteus* were treated by the Lys–MATyr cryogel and Lys–MATyr–clay cryogel. After this process, cell suspensions of *S. aureus* and *M. luteus* were counted with a dilution (1 : 9 dilution rate) plate method with three replications. In the method, decreases in the total cell number were determined by the average number of the colonies after incubation at 37°C for 24 h (Table II). The data in Table II show that the *M. luteus* total cell number decreased from 1.82×10^7 to 9.9×10^6 cfu/mL after interaction with the Lys–MATyr cryogel and the *M. luteus* total cell number decreased from 1.82×10^7 to 1×10^5 cfu/mL after interaction with the Lys–MATyr–clay cryogel. This showed that these two polymeric cryogel materials, which included Lys in their structures, showed antibacterial effects on *M. luteus*. When the results for *S. aureus* were analyzed, it was seen that the total cell number of *S. aureus* decreased from 1×10^8 to 7.4×10^6 cfu/mL after interaction with the Lys–MATyr cryogel and the total cell number of *S. aureus* decreased from 1×10^8 to 1.01×10^7 after interaction with the Lys–MATyr clay cryogel. Also, these values indicated that both the Lys–MATyr cryogel and Lys–MATyr clay cryogel had antibacterial effects on *S. aureus*.

Because of microbial contamination on the materials, all of the materials were sterilized by UV light. Each material was weighed (MATyr–Lys–clay = 0.13 g, MATyr–Lys = 0.2 g), and then, two equal weights were divided into sterile fresh tubes. Nutrient broth (5 mL) was added to each tube. Then, one series of these tubes was inoculated with *E. coli* from the overnight culture, and the other series was inoculated with *S. aureus* from the overnight culture. An uninoculated tube (5 mL of nutrient broth) was used

TABLE II
Colony Number of Bacteria after Treatment with Antibacterial Cryogel Materials

Type of sample	Colony number of *M. luteus* (cfu/mL)	Decrease (%)	Colony number of *S. aureus* (cfu/mL)	Decrease (%)
Original culture	1.8×10^7		1×10^8	
MATyr–Lys	9.9×10^6	45.6	7.4×10^6	92.5
MATyr–Lys–clay	1×10^5	99.45	1.01×10^7	90

as a blank. For the positive control, *S. aureus* and *E. coli* were inoculated into nutrient broth tubes. After incubation at 37°C for 24 h, all tubes were mixed by a vortex. The supernatant was taken and measured at 540 nm, together with the negative controls (blank) and positive controls (absorbance for *S. aureus* = 2.139, absorbance for *E. coli* = 2.068). Decreases in the total cell mass were determined according to the changes in turbidity. The decrease in *S. aureus* was determined to be 2.000 for both of the two materials. Although the turbidity of *E. coli* and the MATyr–Lys–clay was measured as 2.134, the turbidity of *E. coli* and MATyr–Lys was measured as 1.793.

CONCLUSIONS

Lys demonstrates antimicrobial activity against a limited spectrum of bacteria and fungi; however, its enzyme activity can be enhanced by certain substances, including ethyleneglycoldimethacrylate (EDTA), butylparaben, and tripolyphosphate, and by some naturally occurring antimicrobial agents.[33] Lys antibacterial action can be broadened by its modification and the formation of polymeric forms. In this study, Lys activity was enhanced by the interaction of Lys with MATyr, which included tyrosine parts in its structure similar to those in the Lys structure. Then, a photosensitive feature was gained by this modified Lys with MATyr by its interaction with poly(MATyr–Ru–MATyr) and a polymeric material that included Lys–MATyr in its interconnected macropores; a cryogel was synthesized as an antibacterial material against both Gram-positive and Gram-negative bacteria. Also, the surface area of the Lys–MATyr cryogel was increased with organoclay, and this cryogel with Lys–MATyr–clay embedded into its macropores was also used as an antibacterial material against both Gram-positive and Gram-negative bacteria. These new antibacterial materials, which included combined properties of Lys–MATyr, which is effective for damaging bacteria cell walls, and cryogels, which have stable, reusable, strong, and interconnected macroporous structure properties, were used against Gram-positive and Gram-negative bacteria in antibacterial studies. In the results of the experiments, we found a 45.6% decrease in *M. luteus* and a 92.5% decrease in *S. aureus* with MATyr–Lys (Table II). In addition, we observed significant decreases with MATyr–Lys–clay, 99.45% for *M. luteus* and 90% for *S. aureus*.

With studies based on the turbidity, it was found that the interaction of cultures with MATyr–Lys–clay caused a decrease in the in turbidity of *S. aureus* from 2.139 to 2.000. When the same procedure was applied for the turbidity determination of *E. coli,* we did not observe a definite decrease. In addition, the interaction of cultures with MATyr–Lys showed that the decrease of turbidity for the *S. aureus* culture was nearly the same as the value of interaction of the cultures with MATyr–Lys–clay. We observed a great decrease in the turbidity for *E. coli* after the interaction of the cultures with Lys–MATyr.

To sum up, according to all of our results, the MATyr–Lys–clay modification embedded into the cryogel was effective on Gram-positive bacteria and the MATyr–Lys modification embedded into the cryogel was so effective on Gram-negative bacteria as antibacterial materials.

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