A review of immobilization techniques to improve the stability and bioactivity of lysozyme

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ABSTRACT
Lysozyme is a naturally occurring enzyme with antimicrobial activity that has gained significant attention for its potential use in a range of industrial applications. Due to its low toxicity, lysozyme has been considered for use in food, packaging, medicine, medical device and cosmetics. In recent decades, researchers have been working towards extending the lifespan and bioactivity of lysozyme for use in these applications. The immobilization of lysozyme on solid supports has shown positive results as demonstrated by increased stability and extended half-life of the enzyme. In this scoping review, the materials and methods utilized for the immobilization of lysozyme and resulting bioactivities will be reviewed and discussed.

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1. Introduction
Lysozyme is a naturally occurring enzyme that is ubiquitous in nature and most abundant in hen egg white (HEW), human tears and saliva (1–5). The enzyme is easily isolated and purified, and has a low molecular weight (14,500 Da). The enzyme has gained significant attention for its potential use as an antimicrobial agent in a range of applications because of its bioactivity and low toxicity. The free enzyme has been considered for use in packaging, medicine, medical device and cosmetics, and notably, hen egg white lysozyme is currently approved by regulatory agencies for use in foods (6, 7). In recent decades, researchers have been working towards extending and improving the half-life and bioactivity of lysozyme for use in various applications. A relatively short lifespan coupled with challenges associated with lysing both gram-positive and gram-negative bacteria are the main limitations that have prevented industry from fully implementing lysozyme as a natural antimicrobial alternative to other synthetic chemicals (8). An area of research that has received much attention is the immobilization on solid supports. The immobilization of lysozyme has shown positive results as demonstrated by increased stability and extended half-life of the enzyme, and research continues to further extend its activity (9–11). Industries such as biomedicine (7), biotechnology (12–17), biopharmacy (18, 19), food (2, 13, 19–23), and cosmetics (24) have implemented the use of immobilized lysozyme. Related, much research has been recently focused on nanobiocatalysts which combine nanoparticles and enzymes in order to improve activity (25–27). Indeed, there are reports of
combining lysozyme with nanomaterials and we have included a discussion of this work in our review.

The antibacterial mechanism of action of lysozyme is known. It is a glycoside hydrolase that hydrolyzes the peptidoglycan chains found in the cell walls of gram-positive and gram-negative bacteria (Figure 1(A)). Specifically, the enzyme hydrolyzes the β-(1,4)-glycosidic bonds between N-acetylglucosamine (NAG) and N-acetylglucosamine (NAM) residues (Figure 1(B)) (3, 28–30). Hydrolysis occurs at the active site of lysozyme which involves the carboxylic acid moieties of glutamate-35 (Glu-35) and aspartate-52 (Asp-52, Figure 1(B)). Glu-35 donates a proton to the glycosidic ether linkage between NAG and NAM creating an oxonium ion which is followed by nucleophilic displacement of the hydroxy NAG and concurrent formation of an ester linkage of NAM to Asp-52. The ester is then hydrolyzed to provide a terminal hydroxy NAM, completing the scission of the glycosidic bond (31).

The cell wall of gram-positive bacteria consists primarily of peptidoglycan and lacks an outer membrane and so gram-positive bacteria are susceptible to hydrolysis by lysozyme. Gram-negative bacteria, on the other hand, have an outer membrane that contains lipopolysaccharide, proteins and phospholipids above the peptidoglycan, and as such are less susceptible to attack (8, 28, 33, 34). The peptidoglycan is protected from enzymatic activity and so lysozyme is unable to lyse gram-negative bacteria as efficiently as gram-positive bacteria (35).

Given its biological profile, there is significant interest in utilizing lysozyme as a natural antimicrobial against both gram-positive and gram-negative bacteria in various applications and products. However, the lifespan of lysozyme tends to be relatively short in solution. By day 10, lysozyme can lose more than 50% of its initial activity (10, 36). The factors affecting free lysozyme activity (temperature, chemicals, processing and complexes) has been extensively reviewed by Cunningham et al. (37) Current research has focused on extending the lifespan and improving the activity of hen egg white lysozyme (HEWL) by immobilization onto different solid supports. This work has shown that immobilization can improve the stability of lysozyme and consequently extend its bioactivity. In contrast to free lysozyme, immobilized enzyme is known to be more stable to temperature and pH values, and more resistant to environmental changes (10). In addition to extending the half-life, the composition of the immobilized lysozyme itself has a significant effect on the level of antibacterial activity (8, 10, 38, 39).

The various immobilization techniques include covalent bonding (14, 38, 40–42), adsorption (3, 12, 43–45), and encapsulation (18, 46–49). Covalent bonding involves the formation of a chemical bond between lysozyme and the immobilization support (38, 50), whereas adsorption involves surface interactions between lysozyme and the solid-support. Surface interactions include electrostatic, ionic and hydrophobic interactions, van der Waals forces, and hydrogen bonding (50). Another method that has been reported for the immobilization of lysozyme is encapsulation. In this method, the enzyme is trapped within the material and leaches out over time (48, 51–54). The application of these immobilization techniques and resulting bioactivity will be discussed in this review.

There are several reasons to be interested in utilizing lysozyme, a natural product, as an antimicrobial. The creation of a novel antibacterial that is a composite of an enzyme and a solid support from renewable feedstocks is in accordance with the Principles of Green Chemistry (55) and is therefore congruent with sustainable development. Sourcing building blocks from renewable materials can be sustained, and not depleting. Moreover, immobilization of free lysozyme introduces heterogeneity which allows easy recovery and reuse in some cases (10), enhancing the sustainability of the method. Given the safety profile of lysozyme itself, there is the potential of developing an antibacterial that is not only safe for humans but also safe for the ecosystem, particularly given the ability of lysozyme to be biodegraded. This is consistent with the development of products that have reduced hazard, both for humans and the environment (56).

This article is a scoping review of journal publications that is focused on the immobilization of lysozyme on solid supports and the corresponding antibacterial or bioactivity of the immobilized lysozyme. The purpose is to provide the reader with an overview of the research that has been conducted in this area and an understanding of the current state of the art. We highlight significant results and the areas that require further research (gaps). Our approach is to provide a description of the work for each surveyed publication, along with a summary of the main points, particularly as it relates to the technique of immobilization, half-life properties, and antibacterial and/or antimicrobial activities of the modified lysozyme. The review is organized according to the materials that have been used to immobilize lysozyme, namely, chitin and chitosan, cellulose, synthetic polymers, minerals, magnetic and non-magnetic metals, graphene and graphene oxide, and alginate. A summary of highlighted results for each section has been provided in Table 1.

It was not our intention to provide a critical evaluation or a comparison of the surveyed reports as this would be challenging, if not impossible. However, a brief conclusion of each section was provided. In many reports, bioactivity was simply used as a surrogate...
Figure 1. (A) Structural comparison between the bacterial cell wall of Gram-positive and Gram-negative bacteria. (B) Upper; Specific cleavage sites on the peptidoglycan chain for lysozyme, which catalyzes the hydrolysis of the β-(1,4)-glycosidic bond between N-acetylmuramic acid and N-acetyl glucosamine. Bottom; Lysozyme’s mechanism of action. In the enzyme-substrate complex, the Glu-35 residue donates a proton (red) to the glycosidic bond, generating an oxonium that is stabilized by Asp-52 via a covalent bond. The enzyme-substrate intermediate complex reacts with a water molecule (light blue), where Glu-35 accepts a proton from the product of hydrolysis and leaves the enzyme unchanged. References: R = cell wall oligosaccharide chain, R’ = cell wall peptide side chain. (C) Progressive effect of lysozyme on the peptidoglycan of bacteria and lytic activity (32). Reproduced with permission from Springer Nature: Bioresour. Bioprocess. ‘The disruptive effect of lysozyme on the bacterial cell wall explored by an in-silico structural outlook’, Primo, E.D.; Otero, L. H.; Ruiz, F.; Klinke, S. and Giordano, W. (2017). http://creativecommons.org/licenses/by/4.0/
Table 1. Tabulation of immobilization methods with highlighted bioactivity results.

| Materials                  | Immobilization Method | Material Detail                                                                 | Highlighted & Selected Bioactivity as Compared to that of Free Lysozyme | Citation                        |
|----------------------------|-----------------------|---------------------------------------------------------------------------------|---------------------------------------------------------------------------|---------------------------------|
| Chitin and Chitosan         | Covalent bonding      | Wet bead immobilization for wine                                                 | Longer half-life, 21 days                                                | Cappannella et al (36)          |
|                            |                       | Chitin nanowhiskers                                                             | Increase against gram-positive (1.5 fold greater) and gram-negative      | Jiang et al (13)                |
|                            | Adsorption            | Modified chitosan                                                               | Active for roughly 140 days                                              | Crapisi et al (64)              |
|                            |                       | Sulfated chitosan that was grafted to a silicon wafer                           | Most of bioactivity preserved                                            | Tan et al (38)                  |
|                            | Encapsulation         | Chitosan-lysozyme-rectorite                                                     | 92.67% activity against *S. aureus*, 45.12% activity against *E. coli*  | Li, X. et al (2)                |
| Cellulose                  | Covalent bonding      | Lysozyme attached to cellulose via a glycin linker                              | Higher against *B. subtilis*, 23.8 (OD × µmol⁻¹) vs. 0.04 (OD × µmol⁻¹) | Edwards et al (66)              |
|                            |                       | Lysozyme-cellulose conjugates                                                   | Higher                                                                      | Edwards et al (15)              |
|                            |                       | Cellulose nanocrystals                                                          | 75% of activity at 4°C                                                   | Abouhmad et al (14)             |
|                            | Adsorption            | Cellulosic acetate mats                                                         | *S. aureus*-93.9%, *E. coli*- 89.9%                                     | Huang et al (67)                |
| Synthetic polymers         | Covalent bonding      | Eupergit® C                                                                     | 20 times greater against gram-positive bacteria *O. oeni* at pH 3         | Zaccghina et al (69)            |
|                            |                       | Ethylene vinyl alcohol copolymers                                               | Equivalent, ~24 h                                                        | Muriel-Galet et al (70)         |
|                            |                       | Knitted PET                                                                     | *E. coli*-74.1%                                                          | Al et al (71)                   |
|                            | Adsorption            | Cellulosic acetate mats                                                         | *S. aureus*-93.9%, *E. coli*- 89.9%                                     | Uddin et al (68)                |
|                            | Encapsulation         | Cationic cellulose nanofibrils (CNF)                                             | Lysed more *E. coli* and *S. aureus* than free lysozyme                  |                                                |
| Minerals                   | Covalent bonding      | Immobilized lysozyme within a silica matrix through glutaraldehyde cross-linking | *S. aureus*- 95.5%, *E. coli*- 89.6%                                    | Li, H. et al (79)               |
|                            | Adsorption            | Halloysite nanotubes (HNT)                                                      | Lysed *E. coli* more effectively                                        | Wang, Y et al (49)              |
|                            |                       | Mesoporous silica                                                              | 95% and higher at pH 10                                                  | Lu et al (80)                   |
|                            |                       | Silica nanotubes                                                               | Immobilizing lysozyme at 150 mg/g of silica~ 90%,                       | Ding et al (81)                 |
|                            | Encapsulation         | Porous calcium carbonate (CaCO₃) with heparin                                  | Almost 100% against *M. luteus*                                          | Shi et al (48)                  |
|                            |                       | CaCO₃ microparticles                                                            | Almost 100%                                                               | Hassani et al (82)              |
| Metals                     | Covalent bonding      | Superparamagnetic beads                                                         | 50% activity against *E. coli*                                           | Wu, J. et al (83)               |
|                            | Adsorption            | Titanium in the presence of albumin                                             | Increased lysozyme activity in presence of albumin                      | Rösch et al (84)                |
| Graphene-based materials   | Covalent bonding      | Multi-walled carbon nanotubes (MWCNT)                                           | Higher activity against *S. aureus*                                      | Merli et al (85)                |
|                            | Adsorption            | Single-walled carbon nanotubes (SWCNT)                                         | 99% against *M. luteus* and *S. aureus*                                  | Horn et al (86)                 |
| Alginates                  | Encapsulation         | Calcium alginate microspheres                                                   | Bioactivity > 100% over a maximum 16 d period                             | Wells et al (39)                |
| Other                      | Covalent bonding      | Pre-treated wool in glutaraldehyde                                             | 43% antibacterial activity towards *S. aureus* conserved after five cycles of use. | Wang, Q. et al (87)            |

measure of enzyme function with little mechanistic interpretation, and there were variable experimental approaches among authors. Put another way, not all studies were focused on antibacterial activity, but rather, provided bioactivity as a measure of retained enzyme activity upon immobilization. This made direct comparisons impossible in terms of selecting best methods or advantages/disadvantages. The majority of
studies measured activity as a percentage derived from absorbance values relative to unmodified free lysozyme, while others reported activity based on the percentage of bacteria lysis or actual absorbance values without the calculation of activity percentage. The typical bacterium used to test lysozyme antimicrobial activity was gram-positive Micrococcus luteus (M. luteus), formerly known as Micrococcus lysodeikticus. Furthermore, in some studies a variety of both gram-positive and gram-negative bacteria were tested alongside M. luteus. Given this variability, it is difficult to compare immobilization techniques. This speaks to a limitation of the review and the need for more comparative studies in the future. In short, we have included the reported antibacterial activities of immobilized lysozyme where the focus was on the development of an antibacterial, and we also included reported bioactivities of immobilized lysozyme when it was used as a measure of retained enzymatic activity after physically changing the enzyme by immobilization. While not exhaustive, our goal was to provide the reader with an overview of the research that has been conducted in this area.

2. Chitin and chitosan

Chitin is a biopolymer found in the exoskeleton and appendages of arthropods and is a component of the cell wall of lower plants, such as fungi and green algae (57). As such, it is the second most abundant carbohydrate in Nature. One derivative of chitin is chitosan, which is produced by chemical deacetylation of the N-acetyl D-glucosamine backbone in chitin. The abundance of both chitin and chitosan, as well as, their low toxicity, high biodegradability and film-forming abilities have made them an ideal material for the immobilization of lysozyme (2). Furthermore, chitosan has been shown to have its own antimicrobial properties due to the free amino groups present in its structure. These amino groups, usually in their protonated form (i.e. ammonium groups), can weaken the cell wall of bacteria due to their cationic nature. Ultimately, the weakened cell wall allows for increased permeability (58, 59). Moreover, the free amino groups of chitosan can serve as functional handles for derivatization and creation of active derivatives of chitosan. Although chitin and chitosan possess antimicrobial activity and an ability to serve as a building block for chemical synthesis, one concern is that the structures of chitin and chitosan are similar to that of peptidoglycan and so are susceptible to lysozyme cleavage, albeit, at a slower rate. Even with this concern, researchers have developed methods for immobilization which has been shown to slow the hydrolysis of these materials (12, 60). The use of chitin or chitosan as a material for the immobilization of lysozyme has potential in the food packing and biomedical fields. For example, protective films for food were made with chitosan which exhibited antioxidant effects for walnuts (61–63).

2.1. Covalent bonding to chitosan

The most common mechanisms reported in literature for the covalent bonding of lysozyme to chitosan are amidation and Schiff base reactions. The amino groups of chitosan (Figure 2(a)) are the key functional groups for immobilization via covalent bonding to lysozyme (3, 88, 89). Covalent bonding was accomplished via amidation of the carboxyl groups of aspartic acid or glutamic acid residues at the C-terminus of the enzyme with the amino groups of chitosan (Figure 2(b)) (90, 91). Immobilization via covalent bonding was also achieved through a Schiff base reaction where lysozyme is first modified with a cross-linker containing a terminal aldehyde which then reacts with the free amine of chitosan to form the Schiff base (Figure 2(c)).

Song, Y. et al. (40) used a different mechanism to covalently bind lysozyme via a Maillard-type reaction to three different solid supports: galactomannan (a polysaccharide) (92), high molecular weight-type chitosan (HMC) and low molecular weight-type chitosan (LMC). The three constructs were then tested for their antibacterial activity towards gram-negative bacteria. HMC-bound lysozyme proved to be the most effective towards lysing Escherichia coli (E. coli). It was also the most stable at different temperatures and under acidic conditions. At 4°C, HMC-bound lysozyme was able to lyse 80% of the E. coli cells while at 37°C, HMC-bound lysozyme exhibited higher antibacterial activity. In comparison, the LMC-bound lysozyme lysed 80% of E. coli cells at 37°C, however, fewer cells at 4°C. Galactomannan-bound lysozyme showed lower antibacterial activity than lysozyme bound to HMC or LMC.

Yuan, S. et al. (93) demonstrated the use of immobilized lysozyme on grafted chitosan on stainless steel medical equipment. The stainless steel surface was first activated by a biomimetic dopamine anchor to provide active amino groups, followed by covalently immobilizing chitosan with glutaraldehyde as a bifunctional linker. The antibacterial activity of the lysozyme-coated stainless steel was tested against the gram-positive Staphylococcus aureus (S. aureus). The researchers found reduced adhesion of the bacteria to the lysozyme-coated stainless steel compared to non-coated steel and critically, most of the bacterial cells that had adhered did not survive. Bacteria typically adheres to surfaces through electrostatic interactions, van der Waals forces, hydrophobic interactions, and a variety of specific receptor-adhesion interactive forces (93). Chitosan covalently bound to lysozyme
creates a layer with a low surface concentration of positively-charged ammonium groups. Thus, there are fewer contacts on the cell membrane and bacteria cannot adhere to the stainless steel surface as easily. This combined, with lysozyme’s antibacterial activity, provides antibacterial activity.

Within the food industry, lysozyme has been used in wine to reduce the need for sulfur-dioxide as an antioxidant. Sulfur dioxide can cause sensitivity for asthmatic wine consumers (94). Liburdi et al. (95) and Cappannella et al. (36) have investigated the use of lysozyme covalently immobilized to chitosan beads for the purpose of killing bacteria that can spoil wine.

Liburdi et al. (95) attempted a multi-point covalent immobilization of lysozyme to chitosan beads with glutaraldehyde as the crosslink between the two. The activity of these lysozyme-doped chitosan beads was measured in both a model system and in white wine. Overall, the studies showed a loss of activity of immobilized lysozyme towards lactic bacteria Oenococcus oeni (O. oeni, gram-positive) as compared to free lysozyme in white wines. The loss of activity was thought to be a consequence of the steric hindrance around the active sites of lysozyme. Despite the loss in intrinsic activity, covalent immobilization of lysozyme improved its stability in white wine and thereby prolonged the antimicrobial effect.

Similarly, Cappannella et al. (36) used a coupling agent that they referred to as Cn that contained aldehyde moieties that can covalently cross-link with both chitosan and lysozyme via Schiff base reactions. These reactions were performed on both dehydrated chitosan beads and beads wetted with water. The wet bead-immobilized lysozyme was most effective in lysing O. oeni in white wines while the dry bead-immobilized lysozyme was most effective for red wines. The main differences between the white and red wines were their acidity and alcohol content as well as other chemicals found in each wine. Conformational changes of the enzyme may have occurred, which could have led to steric hindrance of the enzyme’s active site that reduced the antimicrobial activity as compared to free lysozyme. Although the activity was reduced compared to free lysozyme, the wet bead-immobilized lysozyme had a longer half-life than free lysozyme (21 days). The
dry bead-immobilized lysozyme had a half-life of 7 days, equal to that of free lysozyme.

Niu et al. (96) designed a study to obtain a washable, nontoxic, safe, and green antibacterial preservation material that can be used for food preservation. They prepared a lysozyme-N-succinyl chitosan (LSZ-NSC) and observed that the lysozyme activity increased by 256% compared with the activity of free lysozyme, and bacteriostatic activity also increased. Following this, they studied LSZ-NSC as a bacteriostatic material in strawberry preservation, and found that it effectively extended the shelf life by 3 days. The group noticed that during the lysozyme loading process, the secondary structure of the enzyme changed, which may be the cause for the change in lysozyme activity.

2.2. Adsorption to chitin and chitosan

Electrostatic interactions between cationic lysozyme and neutral unmodified chitin (97), or cationic chitosan, are minimal. (12, 98). However, the two immobilization materials can be modified with anionic sites to increase electrostatic interactions (38, 99). The primary interaction for this modification is in the form of ionic bonding. Another form of surface interactions is hydrogen bonding. It can occur between the hydroxyl groups of chitosan and the amino groups of lysozyme (2, 65, 98).

2.2.1. The immobilization of lysozyme on chitin through adsorption

The adsorption of lysozyme onto chitin nanowhiskers (CHNW) and its effect on the antibacterial activity of the adsorbed enzyme was studied by Jiang et al. (13). Using a potassium phosphate buffer solution, the lysozyme was adsorbed onto the CHNW and the structural changes were analyzed. The researchers demonstrated that the lysozyme/CHNW system had an improved antibacterial activity that was 1.5-fold greater than that of free lysozyme against the gram positive bacteria Staphylococcus aureus and Escherichia coli. Furthermore, the antimicrobial activity towards E. coli, S. aureus, and Bacillus subtilis (B. subtilis, gram-positive) was greater than that of free lysozyme. The authors suggested that the higher antimicrobial activity towards the gram-negative bacteria was due to the change in the net charge of the lysozyme/CHNW system rather than the conformational changes of the lysozyme itself.

2.2.2. The immobilization of lysozyme on chitosan through adsorption

The adsorption of lysozyme to modified chitosan was studied by Crapisi et al. (64). The chitosan was modified with silica gel on a cross-linked-polystyrene divinylbenzene matrix (Deacidite KMP). The chitosan and silica gel were first combined in a phosphate buffer. Deacidite KMP was then equilibrated by phosphate buffer, followed by lysozyme being stirred into the solution. The results showed that although immobilized lysozyme exhibited a lower activity than free lysozyme, the immobilized lysozyme remained active for roughly 140 days between 5% and 10% activity. The lytic activity fell below 50% at day 7 towards M. luteus.

Tan et al. (38) studied the adsorption of lysozyme onto sulfated chitosan that was grafted to a silicon wafer. The negatively charged sulfate groups of the chitosan likely aided in the electrostatic interactions with lysozyme (99). The lysozyme-chitosan complex effectively lysed the bacterial cell walls of both E. coli and S. aureus. The antibacterial activity of the complex was comparable to that of free lysozyme, and so it was concluded that most of the bioactivity was preserved after this type of immobilization technique.

Li, X. et al. (2) developed a film from chitosan which had enhanced strength and good antimicrobial properties after the addition of lysozyme and rectorite (REC) to the system. Rectorite is a layered silicate that does not possess antimicrobial activity itself but does allow for the adsorption of bacteria onto its surface along with lysozyme. Moreover, REC can improve the strength and thermal properties of composites. A solution of chitosan was used to make three separate films. The first film contained chitosan alone. The second film was adding lysozyme to the chitosan solution before the films were cast and the third film contained chitosan, lysozyme and rectorite, both added before the casting of the film with chitosan. The three films that were cast were compared to one another for their antimicrobial activity against E. coli (gram-negative) and S. aureus (gram-positive). The reported results for the E. coli study comparing chitosan, chitosan-lysozyme and chitosan-lysozyme-rectorite were 23.12%, 33.15% and 45.12% antimicrobial activity, respectively. The chitosan-lysozyme-rectorite film showed the greatest activity. The reported results for the S. aureus study comparing chitosan, chitosan-lysozyme and chitosan-lysozyme-rectorite were 64.15%, 88.15% and 92.67% antimicrobial activity, respectively. The chitosan-lysozyme and chitosan-lysozyme-rectorite films showed similar activities. Comparison to free lysozyme was not reported and the duration of antimicrobial activity was not reported.

2.3. Encapsulation within chitosan

Similar to the adsorption techniques for chitosan, there are reported encapsulation methods involving ionic (53) and electrostatic interactions (12, 65, 100).
Lysozyme encapsulated in chitosan nanoparticles was demonstrated by Deng et al. (46) using ionic gelation technology. Ionic gelation involves the interaction between chitosan’s positively charged ammonium groups and a polyanion, which in this case was sodium tripolyphosphate (TPP) (101). Chitosan and lysozyme were dissolved in various concentrations of acetic aqueous solution followed by the addition of TPP. The process provided encapsulated lysozyme within nanoparticles of chitosan. At a concentration of 4 mg/mL of TPP, lysozyme remained active towards M. luteus for 14 days, with a final activity of approximately 95%. The authors suggested that the high activity retention was likely due to lysozyme being preserved by the acidity of the solution it was dissolved in, which ranged from pH 4.5–5.0. When the chitosan concentration was increased to 1.5 mg/ml from 0.5 mg/mL, the lysozyme encapsulation efficiency also increased.

Wu, T. et al. (33) reported a method of ionic gelation that was similar to that of Deng et al. (46) The difference between the two methods was a higher ratio of chitosan to TPP (3:1) was used by Wu, T. et al. Notably, the authors observed antibacterial activity against gram-negative bacteria, specifically, E. coli, in addition to activity against gram-positive B. subtilis for lysozyme-loaded chitosan nanoparticles. The observed antibacterial activity was higher than the activity of chitosan alone or chitosan based nanoparticles.

Zhang, Y. et al. (100) also reported the encapsulation of lysozyme in covalently cross-linked chitosan-based hydrogels made with difunctionalized PEGs (DF-PEG). Hydrogels are formed through the covalent and noncovalent cross-linking of polymer chains. Noncovalently cross-linked hydrogels are favored in biomedical applications as they are more easily injectable and reversible, unlike hydrogels that are covalently cross-linked. Zhang’s group created the hydrogel by first attaching 4-formyl-benzoic acid to the terminal hydroxyl groups of PEG via esterification reactions. The chitosan was then cross-linked to the aldehyde moieties via a reversible Schiff base reaction involving the free amino groups of chitosan, creating a dynamic hydrogel. Incubation of the lysozyme with the hydrogel provided encapsulated enzyme which was tested against M. luteus in the presence of a stimulator (papain) that promoted lysozyme release. The encapsulated lysozyme retained almost 100% bioactivity upon release relative to a lysozyme-chitosan aqueous solution that was chosen as the control. That activity then increased to ca. 120% after the hydrogel was completely dissolved. The various studies conducted in this research showed that although papain reduces free lysozyme bioactivity, it is possible that as papain digested the chitosan, the damage that would usually occur on lysozyme were counterbalanced.

The Zhang, X. et al. (65) method for enzyme delivery involved encapsulating lysozyme in carboxymethyl chitosan-poly(amideamine) dendrimer core–shell nanoparticles for intracellular lysozyme delivery. The chitosan nanoparticles loaded with lysozyme were formed via electrostatic interactions. The dendrimers almost completely inhibited lysozyme’s activity at physiological pH while it was encapsulated. However, at a lower pH of 5.1, free lysozyme was released from the nanoparticles which maintained its enzymatic activity against M. luteus.

Piras et al. (12) encapsulated lysozyme in chitosan nanoparticles (LZ-NP) using ionotropic gelation. As a result of encapsulation, the lysozyme was active against gram-positive Staphylococcus epidermidis (S. epidermidis) for 5 days. The loading of the lysozyme onto chitosan nanoparticles was considered low because of lysozyme’s low solubility in the aqueous medium where the nanoparticles were formed. There were also scarce electrostatic interactions that created positive charges on both chitosan nanoparticles and lysozyme. However, in the slightly acidic conditions of this research in which LZ-NP were incubated at 37°C, it was determined that the LZ-NPs may provide a controlled and long lasting release of LZ molecules, providing a continuous antibacterial activity. Lysozyme-loaded chitosan nanoparticles killed more colonies of bacteria than chitosan and lysozyme alone. The results support the notion that the system may have possible application for controlled drug delivery (12).

Similarly, Wang et al. (102) aimed to enhance lysozyme’s characteristics via immobilization by encapsulating the enzyme with chitosan nanoparticles. To achieve this, an ionic gelation technique was used. They found that the encapsulated lysozyme demonstrated an effective activity around 70.8 ± 3.6% compared to that of free lysozyme. Furthermore, they concluded that the slight decrease in activity was due to a slight change in the α-helix content of lysozyme, which affected its structure and function. Nonetheless, they found that the encapsulated lysozyme was enhanced in its thermal stability, reusability (retained 71.1% activity after 8 cycles) and its antibacterial activity towards both gram-positive and gram–negative bacteria compared to that of free lysozyme. The encapsulated bacteria showed improved activity towards Pseudomonas aeruginosa (P. aeruginosa), Klebsiella pneumoniae (K. pneumoniae), Escherichia coli (E.coli), and Staphylococcus aureus (S.aureus).
2.4. Summary of immobilization techniques using chitin and chitosan

In this section, the use of chitosan as a solid support for covalent bonding of lysozyme is reviewed. In the studies that reported comparisons of activity to free lysozyme, it can be concluded that the covalent bonding of lysozyme to chitosan typically lowers its antimicrobial activity. This is most likely due to conformational changes and steric hindrance around the active site of lysozyme. However, covalent bonding of lysozyme to the chitosan does tend to extend the lifetime and stability as shown in some cases. This is likely due to conformational changes which can protect the enzyme from external conditions. Research has also shown that lysozyme covalently bound to chitosan has the potential of lysing both gram-positive and gram-negative bacteria which is significant.

We also reviewed adsorption as a technique for the attachment of lysozyme to both chitin and chitosan. It appears that there has been more research demonstrating the success of this approach for the lysis of both gram-positive and gram-negative bacteria. It is also notable that the duration of the lifespan of lysozyme increased, in some cases up to 140 days. Moreover, there is an example of where the activity of lysozyme increased relative to that of free lysozyme after immobilization through adsorption. This is an important observation in terms of commercial applications of antibacterials. In this example, it is thought that the surface interactions related to the adsorption caused fewer conformational changes in lysozyme compared to the changes resulting from covalent interactions.

As for encapsulation, there are literature reports that compared the activity of encapsulated lysozyme to free lysozyme. The authors report evidence of little to no loss of activity after encapsulation. In some cases, activity after encapsulation was higher for both gram-positive and gram-negative bacteria. However, the half-life appeared to be shorter than that of adsorbed lysozyme on chitin and chitosan.

3. Cellulose

Cellulose is the most abundant material on earth and is the primary constituent of plants. It is also found in bacteria, fungi, algae and within animals (14, 103). The structure of cellulose is fibrous, strong and insoluble in water, which gives it the ability to protect plant cell walls. It is composed of beta-D-glucopyranose linked by 1→4 glycosidic bonds, similar to the structure of peptidoglycan, as well as, chitin (Figure 3).

Although cellulose does not possess the antimicrobial properties of chitin or chitosan, researchers have found that cellulose is a suitable carrier for lysozyme in various applications (14–16). The symbiotic interactions of cellulose with lysozyme and other additives can stabilize the complex and thereby extend antimicrobial activity (104). Other advantages to using cellulose as the support for lysozyme include its availability, renewable origin, low cost, ease of modification, biocompatibility and biodegradability (16, 68, 103, 105–107).

3.1. Covalent bonding of lysozyme to cellulose

Several synthetic methods have been developed to covalently bind lysozyme to cellulosic materials. A common method is creation of amide linkages between lysozyme and cellulose. Edwards et al. (66) used 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) as the coupling agent, the amino groups of glycine-esterified cellulose and the carboxylic groups of aspartate and glutamate residues in lysozyme are cross-linked via amidation reactions (Figure 4). The antibacterial activity of the cellulose-
bound lysozyme was assessed over 5 h. Activity was 'measured as a ratio of optical density change (OD) over the total number of micromoles of lysozyme assayed per microtiter well.' (66) The activity was measured to be 23.8 (OD × µmol⁻¹), at its highest, when lysozyme was attached to cellulose via a glycine linker. Free lysozyme exhibited the lowest activity towards the gram-positive B. subtilis at 0.04 (OD × µmol⁻¹). The results demonstrate that lysozyme can not only maintain its activity when coupled to cellulose via glycine linkages, but can also improve its activity (66). The authors believe that the enhanced stability of the enzyme was due to the covalent binding of the enzyme to the cellulose, a solid-phase support. Furthermore, x-ray crystallography showed that lysozyme's active site was accessible after immobilization.

Edwards et al. (16) later expanded on this work by exploring the use of other glycine-derivatized cotton fabrics, which included woven print cloth and nonwoven fabric. The fabrics were crosslinked with lysozyme using citric acid and aminosilane coupling agents to produce materials with antibacterial activities. The highest antimicrobial activity against M. luteus was observed for lysozyme that was cross-linked to nonwoven cotton with citrate. There was no comparison of the activity of lysozyme bound to fabric to that of free lysozyme; however, the authors did include a figure showing the enzymatic activity of free lysozyme at different concentrations.

Edwards et al. (15) continued their work on carbodiimide-activated coupling reaction of lysozyme to glycine-cellulose. The authors immobilized lysozyme to cotton nanocrystals (NC), specifically NCI and NCII. The NCI had a ribbon shape that included voids throughout while NCII appeared to be densely packed particles that had been broken off from a sheet. The authors proposed that increased surface area would provide increased antimicrobial activity, and indeed both the lysozyme-cellulose conjugates (NCI and NCII) demonstrated higher antibacterial activity towards M. luteus than free lysozyme. Edwards and co-workers (108) then studied different cotton materials, for example, cotton twill and cotton spunlace, in order to determine which material was best for retaining lysozyme activity. Both NCI and NCII showed similar activities that were regarded as high compared to free lysozyme, albeit lower than free lysozyme.

Datta et al. (109) covalently immobilized lysozyme to p-aminobenzyl (PAB)-cellulose fibers through a modified diazonium intermediate procedure (110). The covalently, immobilized lysozyme on polyacrylamide retained an appreciable fraction (about 50%) of its enzymatic activity towards M. luteus (109). The authors note that it was difficult to differentiate between the activity of soluble and immobilized lysozyme when cellulose was used as a support. A comparison to free lysozyme was not discussed.

Abouhmad et al. (14) studied different materials, including cellulose, and different immobilization techniques (adsorption versus covalent bonding). Their results showed that cellulose nanocrystal-bound lysozyme had the highest lytic activity towards gram-positive and gram-negative bacteria as compared to free lysozyme due to the overall positive charge of the complex. The immobilized lysozyme preparations were more stable than free lysozyme when stored at 4 and 22°C. Moreover, cellulose nanocrystal-bound lysozyme maintained 40% of its activity after adsorption compared to 50% of its activity after covalent bonding to cellulose at 22°C. At lower temperature (4°C), the activities were at 60% and 75%, respectively. The duration of these tests lasted 60 days. In comparison, free lysozyme lost its activity after 20 days at 22°C and 35 days at 4°C.

Xue et al. (111) reported the preparation of novel, carboxyl-functionalized core–shell magnetic cellulose microspheres (MCMS) by surface modification with 1,2,3,4-butanetetracarboxylic acid (BTCA) and the immobilization of lysozyme via covalent bonding. The authors found that the immobilized lysozyme showed excellent activity within wide pH and temperature ranges as well as the high storage and thermal stabilities compared to free lysozyme (111).

### 3.2. Adsorption of lysozyme to cellulose

In literature, the adsorption of lysozyme to cellulose has been reported to involve electrostatic interactions (67, 112) and ionic bonding (43, 113).

Gemili et al. (112) prepared cellulose acetate based antimicrobial films that contained lysozyme by using a dry phase inversion technique in an effort to develop novel food packaging materials for the controlled release of the antimicrobial agent. They blended lysozyme into a cellulose acetate solution to form the antimicrobial films. The authors noted that the presence of acetone reduces the dielectric constant of the medium which favors electrostatic interactions. However, these interactions can also cause changes in the conformation of the enzyme and the diffusion of acetone into interior parts of the proteins above 0°C may disrupt the hydrophobic interactions within the enzyme. Gemili et al. determined that the decrease in porosity, release rates, maximum activity of released lysozyme, and antimicrobial activities led to an increase in lysozyme activities and tensile strength of the films when cellulose acetate content in the solution was high. Antibacterial
activity was tested against *M. luteus*, gram-positive *Bacillus amyloliquefaciens* (B. amyloliquefaciens), and *E. coli*. Activity comparisons to free lysozyme were not reported. According to the authors, the highest release rate and antimicrobial activity of the lysozyme were obtained when the films were prepared from 5% cellulose acetate solution with 1.5% lysozyme (112). Furthermore, by adding disodium ethylenediaminetetraacetate, which is capable of lysing gram-negative bacteria, the films became effective towards *E. coli* and were most effective when combined with lysozyme.

Mascheroni et al. (43) demonstrated that manipulation of the concentration of anionic carboxymethyl cellulose in paper matrices allowed for an increase in adsorption of lysozyme through ionic bonding with the charged paper fibers. The ratio of short to long paper fiber were adjusted for maximum antimicrobial activity. The desorption of lysozyme from the paper matrix was analyzed and the antimicrobial activity tested against *M. luteus*, gram-positive *Listeria innocua* (L. innocua), and a gram-negative bacteria, *E. coli*. Desorption of lysozyme was triggered by humidity and the rate of desorption increased with medium to high porosity. The best antibacterial activity was observed using a 50/50 ratio of short to long paper fibers with 3.8% carboxymethylcellulose. Both tests showed higher activity towards bacteria than the paper without lysozyme. A comparison of antimicrobial effects of free lysozyme was not discussed.

This area of study was continued by Barbiroli et al. (113) who attempted to improve the antimicrobial activity of lysozyme towards gram-negative bacteria by incorporating a whey glycoenzyme, lactoferrin. Lactoferrin increases the permeability of the outer membrane of gram-negative bacteria by depriving bacteria of iron, and by interacting with the bacteria’s anionic lipopolysaccharide layer with the cationic patches on the surface of lactoferrin. These alterations caused by this interaction results in the release of lipopolysaccharide. Lactoferrin can also affect gram-negative bacteria by decreasing the cell wall’s negative charge which exposes the peptidoglycan layer to lysozyme. The lysozyme-lactoferrin construct prolonged the lag phase of *L. innocua* which is the first phase of the bacterial growth cycle. The lag phase duration increased to 6.5 h and *E. coli*’s lag phase duration increased to 2.68 h. Overall, when these materials were applied on actual meat samples under similar conditions of practical use, results demonstrated lysozyme’s efficiency in preventing growth of *Listeria* (113).

Huang et al. (67) used a layer-by-layer system involving cellulose acetate mats. Positively charged N-[(2-hydroxy-3-trimethyl-ammonium) propyl] chitosan chloride (HTCC) with immobilized lysozyme and negatively charged alginate were applied as alternating layers. The layers were created by electrostatic interactions. The antibacterial activity of the layered system could be increased by increasing the number of layers applied to the cellulose acetate mat, most likely due to the increasing concentration of lysozyme within the material. Additionally, if the lysozyme-HTCC was placed as the outermost layer (and not the alginate), a higher antibacterial effect towards gram-negative bacteria was observed. The coated cellulose acetate mats also showed stronger lysing for gram-positive bacteria with each tested concentration. Activity for *S. aureus* was 93.9% and activity for *E. coli* was 89.9% relative to free lysozyme. Additionally, immobilized lysozyme showed higher stability towards environmental changes, such as temperature and pH, than free lysozyme and can could be reused, however, the duration of activity was not discussed.

Uddin et al. (68) used different forms of cellulose nanofibrils (CNF) to create nanocellulose aerogels for lysozyme adsorption. Untreated CNF and treated CNF with 2,2,6,6-tetramethylpiperidine-1-oxyl-sodium bromide-sodium hypochlorite (TEMPO-NaBr-NaClO) were used in the study. Lysozyme adsorption was accomplished via electrostatic interactions. To test antibacterial activity, the lysozyme containing aerogels were plated with *E. coli* and *S. aureus* for cell counting. Changes in antibacterial activity and stability were observed over 30 days. As TEMPO-oxidized CNF is negatively charged, adhesion of the positively-charged lysozyme was stronger than the other CNF aerogels. However, alteration of the lysozyme structure resulted in a lower activity as compared to other CNF aerogels. However, cationic CNF–lysozyme was able to lyse more *E. coli* and *S. aureus* than free lysozyme. The observed activity decreased slightly over 30 days.

### 3.3. Summary of Immobilization techniques using cellulose

The covalent immobilization of lysozyme to cellulose showed similar results to that of chitin and chitosan. It was found that lysozyme went through conformational changes, most likely due to the cross-linkers that were used in the chemical bonding. Gram-negative bacteria did not have a significant increase of susceptibility to lysozyme covalently bound to cellulose. Notably, the activity of covalently immobilized lysozyme towards gram-positive bacteria was relatively high compared to that of free lysozyme. Notably, stability was shown to increase in some cases as the half-life of lysozyme was extended.

Lysozyme that was adsorbed to cellulosic materials showed good antibacterial effects towards both gram-
positive and gram-negative bacteria. Stability was also increased. However, those effects were lower for gram-negative bacteria than that observed for gram-positive bacteria.

Finally, it should be noted that cellulose does not possess its own antibacterial properties, unlike chitin and chitosan. Further research is needed to have extensive comparisons of activity to free lysozyme and measurement of activity for the immobilized lysozyme over time.

4. Synthetic polymers

Polymer-immobilized lysozyme has the potential to be used for water treatment, food manufacturing (19), and biomedical (71) applications. There are multiple advantages for selecting synthetic polymers for the immobilization of lysozyme. In addition to being a carrier, the polymer itself can contribute to the function of a material and its surface can be modified in order to increase antibacterial or antimicrobial activity.

Polymers that are grafted onto a material can act as intermediates for chemical modifications. That is, the polymer can display different chemical groups for functionalization. For example, polymers can be designed to contain quaternary ammonium groups (R-NH₃⁺) which would cause the polymer to have its own antimicrobial activity (Figure 5). The toxicity of quaternary ammonium groups is a result of their ability to exchange Ca²⁺ and Mg²⁺ in the bacterial cytoplasmic membrane which destabilizes the bacterial intracellular matrix. Quaternary ammonium groups can be prepared with hydrophobic tails that interlace over the bacterial surface which causes leakage of intracellular fluid from the cytoplasmic membrane (114). This is a similar attribute to the structure of chitosan as it can also form antibacterial cationic groups through the free amino functional groups (58). Lastly, polymer surface modifications can facilitate interactions with bacteria, such as bacterial adhesion to the surface which could increase the antibacterial activity effect of the polymer (41, 71, 115–117).

4.1. Covalent bonding to polymeric materials

4.1.1. The immobilization of lysozyme on ethylene-based surfaces through covalent bonding

Zacchigna et al. (69) studied the covalent bonding of lysozyme to Eupergit® C, a polymeric solid support consisting of macroporous beads (Figure 6). The lysozyme was immobilized to the support via long and flexible polyethylene glycol (PEG) linkers. The length of the PEG spacers was varied in order to increase diffusion rate and minimize steric hindrance of the carrier (69). When considering the effect of immobilized lysozyme on *M. luteus* and *Leuconostoc oenos* (*L. oenos*), it was found that the optimization of the PEG chain length lead to higher activity than that seen for free lysozyme. The PEG-lysozyme construct demonstrated an increase in enzymatic activity that was 20 times greater than

![Figure 5](image-url). Polymer containing positively-charged quaternary ammonium groups destabilizing a bacterial cell membrane via attractive forces with negatively charged phospholipids (118). Reprinted from Food Packaging and Shelf Life, Vol 12. Kedafi Belkhir, Monique Lacroix, Majid Jamshidian, Stéphane Salmieri, Corinne Jegat, Mohamed Taha. Evaluation of antibacterial activity of branched quaternary ammonium grafted green polymers. Pages 39. Copyright © 2017 with permission from Elsevier with edits.

![Figure 6](image-url). Immobilization of lysozyme via PEG spacers onto Eupergit C via covalent bonding, re-drawn. (69) Reprinted by permission from Springer Nature: Applied Biochemistry and Biotechnology 'Improved activity in acidic media of immobilized lysozyme', Zacchigna, M. et al. (69).
that of free lysozyme towards the gram-positive bacteria L. oenos at a pH of 3.

Caro et al. (21, 22) focused on protecting stainless steel surfaces from bacterial and enzyme adhesion by first creating a Cr-enriched, hydroxylated oxide top layer on stainless steel coupons (SS-SC) followed by pre-treating these coupons with amino-enriched polyethylene imine (SS-SC-PEI). This was then followed by grafting PEG and/or lysozyme onto the surface (Figure 7). Spontaneous physisorption of PEI on stainless steel provides a surface layer of amino groups. The HEWL (lysozyme obtained from egg whites) was attached to the stainless steel surface via two, sequential, reductive amination reactions. The stainless steel pretreated with PEI was reacted with glutaraldehyde in the presence of NaCNBH₄, followed by treatment with lysozyme, again in the presence of NaCNBH₄. The two step procedure provided grafted lysozyme. To graft PEG, it was bound to PEI’s amino-terminated side chains via a reductive amination reaction. This was achieved by immersing SS-SC-PEI substrates overnight in a phosphate buffered saline (PBS) solution of PEG dialdehyde and NaCNBH₃. Finally, cогrafting PEG and lysozyme involved treating SS-SC-PEI-PEG surfaces with glutaraldehyde and lysozyme. PEG was grafted first due to the heat required for its immobilization that could have denatured the lysozyme. It was noted that there was a possibility of grafting lysozyme on the aldehyde end of either PEG or glutaraldehyde during these experiments. Through this research, Caro et al. (21, 22) observed that grafted PEG, grafted lysozyme, or co-grafted PEG and lysozyme onto the stainless steel surfaces coated with PEI led to significant decreases in adhesion of proteins or bacteria. Notably, co-grafting of PEG and lysozyme also showed long term antimicrobial activity against M. luteus and gram-positive Listeria ivanovii (L. ivanovii).

Caro et al. (46) further observed that the antimicrobial activity of lysozyme could be increased by immobilizing the enzyme further away from the surface of the stainless steel by cross-linking an extra layer of PEI by glutaraldehyde to the structure (SS-SC-PEI-GA-HEWL). The added distance between lysozyme and the stainless steel surface increases lysozyme’s accessibility. This was compared to their previous work, mentioned above, where lysozyme’s active site was sterically hindered.

Muszanska et al. (119) constructed polymer-protein conjugates that were adsorbed onto gold-coated quartz surfaces to create functional coatings for biological systems, such as biomaterials implant surfaces. The polymer-protein conjugates consisted of lysozyme as the protein and a synthetic co-polymer made from one unit of polypropylene oxide (PPO) and two units of polyethylene oxide (PEO). The lysozyme was attached to the co-polymer via reductive amination of the aldehyde functionalized PEO blocks and the amine groups of lysine residues of the lysozyme. Testing the antibacterial activity of the lysozyme-PEO system against B. subtilis showed that it had a slightly lower antibacterial activity than that of free lysozyme. Some activity loss for lysozyme-PEO was possibly due to conformational changes of lysozyme as a result of the covalent coupling.

Muriel-Galet et al. (70) used two different ethylene vinyl alcohol copolymers (EVOH 29 and EVOH 44), which were modified with UV irradiation treatment to generate carboxylic groups. Afterwards the film was treated with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and lysozyme which resulted in covalently bound lysozyme to the co-polymer (Figure 8). Gram-positive Listeria monocytogenes (L. monocytogenes) was used to test antimicrobial activity. The antimicrobial properties of this immobilized lysozyme was equivalent to that of free lysozyme and lasted for approximately 24 h. However, activity was significantly reduced after this

Figure 7. Schematic representation, with minor edits, of the stainless steel (SS) surfaces functionalized by PEI-GA-HEWL (a) and by PEI-PEG (b) shows the product of reductive amination (45). Reprinted (adapted) with permission from Caro et al. (21). Copyright © 2009 American Chemical Society.
period to a mean activity of 120 and 125 units/mg for lysozyme immobilized on EVOH 29 and EVOH 44, respectively. The authors proposed that the activity loss could be from enzyme denaturation, limitations to substrate accessibility due to enzyme orientation, steric hindrance, or diffusion limitations.

Al et al. (71) covalently immobilized lysozyme onto woven and knitted crimped polyethylene terephthalate (PET) grafts in order to prevent bacterial adhesion of broad spectrum pathogens. Lysozyme was attached to the PET grafts through what the authors called the broad spectrum pathogens. Lysozyme was attached to PET grafts in order to prevent bacterial adhesion of woven and knitted crimped polyethylene terephthalate (PET) grafts against lyses between lysozyme bound to woven and to knitted PET were as follows: for M. luteus the activities were 58.4% and 55.9%, respectively. For S. aureus, the activities were 81.5% and 80.8%, respectively. For S. epidermidis, the activities were 84.5% and 87.8%, respectively. Finally, for E. coli the activities were 73.3% and 74.1%, respectively. These values are relative to that of free lysozyme.

Another polymer of interest in this field is silicon rubber which is a material often used in the medical device industry. Flores-Rojas et al. (72, 120) studied lysozyme that was covalently immobilized onto silicon rubber (SR) by grafting polyethylene glycol dimethacrylate-co-glycidyl methacrylate p(EGDMA-co-GMA) to the silicone rubber by gamma-ray radiation to provide a formyl group for lysozyme immobilization. The first paper reported by this group (120) covered two methods. Method 1 required a reduction of imine groups to form the acetal group that would then be hydrolyzed into a formyl group. Method 2 achieved its formyl group via reduction of nitrile groups to create the necessary acetal group for hydrolysis into a formyl group (Figure 9). Method 1 showed the highest lysozyme activity retention compared to Method 2 at pH 7. However, neither method retained as high of an activity as that of free lysozyme (120).

In the second paper by Flores-Rojas et al. (72), they achieved the resulting formyl group for lysozyme immobilization through the use of azobisisobutyronitrile (AIBN) as an initiator for the polymerization of EGDMA and GMA monomers onto the silicon rubber rather than using gamma-ray radiation (Figure 10). This work demonstrated improved lysozyme activity at a higher temperature and lower pH value compared to that of free lysozyme (72).

4.1.2. The immobilization of lysozyme on amide based surfaces through covalent bonding

Saeki et al. (41) investigated the immobilization of lysozyme to polyamide reverse osmosis (RO) membranes. Lysozyme was covalently immobilized on the surface of the RO membranes by an interfacial polymerization method. The method involves a polymerization reaction between 1, 3,5-benzenetricarbonyl trichloride and 6-amino caproic acid (ACA). ACA was used as a spacer to minimize steric hindrance between lysozyme and the membrane. Lysozyme was then immobilized to the ACA-modified polyamide layer through an amine coupling reaction with EDC and N-hydroxysuccinimide (NHS). The immobilized lysozyme was tested for antibacterial activity against two gram-positive bacteria, B. subtilis and M. luteus. The results indicated that the B. subtilis was completely lysed and the antibacterial activity of the membrane lasted up to 5 months, although it decreased by 30% compared to that of free lysozyme likely due to denaturation of the immobilized lysozyme via hydrolysis.

4.1.3. The immobilization of lysozyme on carbon-based surfaces through covalent bonding

Wang, J. et al. (121) immobilized lysozyme on three-dimensional ordered mesoporous carbon composite in a two-step chemical synthesis. The composite was activated by EDC, creating a reactive intermediate that reacted with NHS giving the corresponding NHS-ester. The NHS-ester was then coupled with a free amino group in lysozyme via an amidation reaction, providing covalently bound lysozyme to the carbon composite. This procedure provided immobilized lysozyme on mesoporous carbon composite that displayed 80% antibacterial activity towards S. aureus.

Dutta et al. (122) immobilized lysozyme on glass surfaces by first modifying the surface with an self-assembled monolayer via cross-metathesis reactions that produced a monolayer with terminal Fischer carbene. The free amino groups of lysozyme were then reacted with the electrophilic carbene moieties to produce immobilized lysozyme. The immobilized...
lysozyme demonstrated a significant lytic activity towards *M. luteus*, however, exact activity was not calculated and a DEAD/LIVE method was used.

4.1.4. The immobilization of lysozyme on gel-forming polymers through covalent bonding

Verheyen et al. (123–125) immobilized lysozyme on a set of hydrogels. These hydrogels were designed to be carriers for drug delivery systems of proteins in an effort to find an improved drug delivery system. In their work, they selected lysozyme as their surrogate protein. Interestingly, the system was designed to release the protein under certain conditions. The observed bioactivity with the system retained 70% of its bioactivity towards *M. luteus* after being tested for 168 h (125). The authors suggested that loss of bioactivity was due to the polymerization process.

4.1.5. The immobilization of lysozyme on other polymers through covalent bonding

Sert et al. (126) used sol–gel chemistry to covalently bond lysozyme to a stainless steel surface. This technique involves the attachment of carboxyl functional groups of lysozyme to a stainless steel surface through a carbodiimide reaction. Another approach utilized by Sert et al. (126) was to covalently bond lysozyme to the stainless steel using the lysozyme’s N-terminus amino groups and the poly(acrylic acid) (PAA) brushes. The PAA brushes functioned as the linker between lysozyme and the stainless steel surface. The method...
provided the material with a 30x fold increase in the amount of lysozyme as compared to the first method. Activity differences were observed and were dependent on the location of covalent immobilization to the lysozyme. Activity was 7.5 times higher when immobilization occurred at the amino-terminus rather than the carboxyl-terminus. The data indicated that immobilized lysozyme in the presence of PAA brushes displayed antibacterial activity but comparisons to free lysozyme were not reported.

Liu et al. (19) covalently immobilized lysozyme on a polyacrylonitrile (PAN) membrane through the use of glutaraldehyde as the cross-linker, specifically for applications in water treatment, food, pharmaceutical, and biotechnological industries. The lysozyme modified membrane demonstrated high antibacterial activity towards *S. aureus*. They found that 56.6% of the *S. aureus* underwent lysis in solution, compared to only 19.3% in the control group. The authors suggested that reduction in activity may be attributed to diffusional limitations from the diminished molecular flexibility of the enzyme. Another reason could be due to enzyme conformational changes and damage to the active site. Preservation of lysozyme stability lasted approximately 3 months in 0.1 M PBS (pH 6.8) solution. The immobilized lysozyme lost 24% of its initial activity likely due to denaturation and hydrolysis.

Costoya et al. (127) investigated the immobilization of lysozyme and acylase 1 onto a poly(vinyl chloride) (PVC) catheter grafted with glycidyl methacrylate (GMA). First, the GMA was grafted onto the PVC catheter (PVC-g-GMA) by a pre-irradiation method followed by enzyme immobilization. It was found that the lysozyme readily reacted with the PVC-g-GMA due to the predominance of basic amino acids, arginine and lysine. The antimicrobial activity of the covalently bound lysozyme was tested against *M. lysodeikticus* at pH 6.22 and it was found that all catheters functionalized with lysozyme showed enzymatic activity that was directly related to the graft percentage of the enzyme. Further tests were performed against the gram-positive bacteria *S. aureus* by incubation of the lysozyme functionalized catheter in a concentrated bacterial suspension. Comparing the viable bacteria adhesion levels to that of a control, it was found that the catheters functionalized with lysozyme decreased the attachment of *S. aureus* to the surface by 2–3 orders of magnitude.

Levashov et al. (128, 129, 130) examined the antimicrobial activity of covalently immobilized lysozyme and demonstrated the differential effects of glycine and...
charged amino acids on the enzymatic lysis of gram-positive and gram-negative bacteria by soluble and immobilized lysozyme (128). The same group studied different methods of covalent lysozyme immobilization and identified a novel method for lysozyme immobilization on a polymeric agarose matrix that provided antibacterial action and eliminated the leakage of the enzyme from the material. The resultant immobilized lysozyme exhibited bacteriolytic activity toward gram-positive bacteria Micrococcus luteus and gram-negative bacteria Escherichia coli (129). In a separate report, they demonstrated for the first time that immobilized lysozyme can efficiently remove E. coli and Pseudomonas aeruginosa endotoxins from solutions (130). The same research group also studied covalently immobilized lysozyme and examined its bacteriolytic activity against E. coli. Chemical modification of lysozyme was carried out using benzaldehyde and anisaldehyde. It was observed that covalent immobilization only affects 1–2 amino groups of the lysozyme which preserved bacteriolytic activity (131).

Mehrabi et al. (132) reported the development of antibacterial membrane surfaces that can mitigate biofilm formation. α-Amylase and lysozyme were covalently immobilized on polydopamine/cyanuric chloride functionalized polyethersulfone (PES) membranes to form biocompatible antibacterial surfaces. The authors reported that the α-amylase/lysozyme mixture treated membrane samples showed greater than 87% removal for S. aureus and S. epidermidis biofilms as indicated by a microtiter test.

4.2. Adsorption to polymeric materials

4.2.1. The immobilization of lysozyme on ethylene based surfaces through adsorption

Conte et al. (117) studied the antimicrobial activity of lysozyme after immobilization on modified polyethylene (PE) films through a plasma treatment process. Plasma treatment of the PE surface with H₂ and H₂O exposed carboxylic acid functional groups. These groups were then available for interaction with the polar amine groups of lysozyme. Adsorption of the lysozyme onto the PE films occurred through electrostatic interactions. All tests showed antibacterial activity of the PE-immobilized lysozyme towards M. luteus. The authors report that the demonstrated activity was due to the bacteria interacting with the surface of the enzyme-loaded polymer rather than the release of lysozyme into the solution. The nature and mechanism of this surface interaction are still being explored.

In another example, lysozyme was immobilized through adsorption onto the polymer poly(3,4-ethylenedioxythiophene) (PEDOT) by Teixeira-Dias et al. (73). Adsorption of lysozyme was achieved via hydrogen bonding to oxygen atoms throughout the polymer. Furthermore, lysozyme was incorporated into the PEDOT film through a second technique referred to as an in situ electrochemical polymerization. However, it was found that lysozyme’s structure was altered because of the hydrogen bonding. The PEDOT-lysozyme material had greater antibacterial activity against gram-positive bacteria (S. epidermidis) than the PEDOT alone and the activity remained close to the activity of free lysozyme, signaling that adsorption did not induce conformational changes. However, the lysozyme-PEDOT and the PEDOT alone had no effect towards gram-negative E. coli.

Yu, Q. et al. (115, 116) adsorbed lysozyme onto poly(N-isopropylacrylamide) (PNIPAAm) brushes that also featured biocidal quaternary ammonium salts (QAS). PNIPAAm brushes are considered ‘smart’ materials due to their ability to respond quickly to temperature changes in their surrounding environment, as well as having the ability to reverse those changes. (115, 116). The changes involved the concealment and exposure of ungrafted substrate between polymer-grafted regions via thermal triggers. The ungrafted regions were used to adsorb lysozyme and provide a switchable surface bioactivity. For this system, both lysozyme and QAS act as biocides towards bacteria and the PNIPAAm polymer brushes control the surface’s interactions with bacteria. Experimentation was done around the lower critical solution temperature (LCST). Above the LCST, PNIPAAm brushes can attach to the bacteria and the exposed QAS kill bacteria. Below LCST, PNIPAAm brushes release dead bacteria. Both gram-positive and gram-negative bacteria were killed with this technique. For E. coli, the killing efficiency was close to 60% for PNIPAAm/lysozyme at 37°C with a minimal killing efficiency loss at 25°C. The killing efficiency was at almost 80% for S. epidermidis at 37°C with a slightly higher decrease of killing efficiency at 25°C than what was seen with E.coli.

4.2.2. The immobilization of lysozyme on polyvinyl alcohol based surfaces via adsorption

Zhan et al. (44) fabricated nanofibrous membranes that were composed of adsorbed lysozyme, polyvinyl alcohol (PVA) and an aluminium phyllosilicate material called rectorite (Figure 11). The membranes were constructed via electrospinning and feature hydrogen bonding between the hydroxy moieties of PVA and rectorite and the free amine and hydroxy groups of lysozyme. The immobilized lysozyme demonstrated antibacterial activity towards both gram-positive
(S. aureus) and gram-negative (E. coli) bacteria. Antibacterial activity was captured by the inhibition zone method. The greatest inhibition zone was roughly 13 mm for S. aureus and 9 mm for E. coli when with 60/40 PVA/LY mass ratios containing 1 wt% lysozyme (LY) exfoliated rectorite (REC). The authors believe that the higher antibacterial activity could be due to the ability of REC to adsorb and immobilize the bacteria on its surface at high capacities. Furthermore, the lysozyme accumulated on the polymer chain of the REC surface could create a larger distance between the enzyme and REC causing the active site to be more accessible to lyse bacteria on the surface.

Tonglairoum et al. (133) developed lysozyme immobilized ion-exchange nanofiber mats to promote wound healing. The authors selected nanofiber mats since it mimics the outer structure of cells that supports cell attachment and proliferation. They studied two nanofiber mats that consisted of poly(styrene sulfonic acid-co-maleic acid)/polyvinyl alcohol (PSSA-MA/PVA), and poly-(acrylic acid-co-maleic acid)/polyvinyl alcohol (PAMA/PVA). The mats were constructed by electrospinning followed by thermal cross-linking. Lysozyme was then immobilized by an adsorption method. Both lysozyme immobilized nanofiber mats retained antibacterial activity and promoted wound healing. It was noted that the lysozyme-PSSA-MA/PVA nanofibers displayed a higher activity with 647.6 ± 58.8 µg/g lysozyme content and 7.62 ± 1.24 IU/g activity against M. luteus compared to lysozyme-PAMA/PVA nanofiber mats with 165.9 ± 11.6 µg/g lysozyme content and 17.88 ± 1.03 IU/g activity. The difference in activity came from the higher loading of adsorbed lysozyme on the PSSA-MA/PVA nanofiber.

4.2.3. The immobilization of lysozyme on gel-forming polymers via adsorption

Lysozyme adsorption onto carbon aerogels (CA) can be applied to areas such as separation, biocatalysis, biosensors and controlled release and delivery (134).

Dekina et al. (74) non-covalently immobilized lysozyme to a mucoadhesive gel. This material is used within the medical field. The gel was made of carboxymethyl cellulose sodium salt (Na-CMC) which interacted with lysozyme through electrostatic forces and hydrogen bonding. The immobilized lysozyme was more effective than free lysozyme in lysing M. luteus at elevated temperatures and higher pHs. Optimal pH was 6.2 while the optimal temperature was 37°C during storage for 180 mins. Storage time was key in the preservation of lysozyme activity in these conditions. The final product remained stable for two years with 95–100% of original retained activity.

Rios et al. (49) loaded lysozyme into polyacrylic acid microgels by suspending the microgels in lysozyme solution containing phosphate buffer solution. This allowed for electrostatic interactions between the positively charged ammonium groups within lysozyme and the anionic dextran hydrogels. A high-loading efficiency was possible as a result of the polyionic interactions between the microgel’s backbone and lysozyme. Notably, this technique allowed for the preservation of more than 90% of lysozyme’s activity towards M. luteus for 30 days.

In another study by Li, Y. et al. (135), lysozyme was trapped inside of a microgel consisting of oxidized potato starch polymers which was chemically cross-linked with sodium trimetaphosphate (STMP). The electrostatic interactions between the negatively charged starch polymer and the positively charged ammonium groups of lysozyme allowed for the uptake into the microgel when the two were combined in solution. It was determined that this antimicrobial system is most effective towards amylase-producing, lysozyme-sensitive bacteria. This research was tested with gram-positive bacteria and results showed that activity was slightly lower than that of free lysozyme.

4.3. Encapsulation within polymeric materials

4.3.1. The immobilization of lysozyme on poly(DL-lactide-co-glycolide) (PLGA) via encapsulation

Nam et al. (136) discovered that adding urea to lysozyme-containing poly(D,L-lactide-co-glycolide) acid (PLGA) microspheres retained 71.3% of lysozyme’s bioactivity over 28 days. They reported that this was likely due to urea’s hydrogen bonding and/or hydrophobic
interaction with the lysozyme backbone which resulted in maintaining the enzyme’s conformation.

Pérez et al. (137) studied the preservation of lysozyme structure and activity after encapsulation in bioerodible polymers via water-in-oil-in-water (W/O/W) techniques. The authors were investigating the effect of adding additives, namely lactose and lactulose, on the stability of the lysozyme microspheres. Lysozyme was encapsulated in PLGA by first dissolving the lysozyme and PLGA with various additives. The emulsions were then poured into solutions of PVA and distilled water and were stirred. Phosphate buffer was slowly added to harden the microspheres (Figure 12). The encapsulation of lysozyme in PLGA microspheres decreased the antibacterial activity against *M. luteus* which was improved to 90% with the addition of lactose and to 99% with lactulose additive. Acetonitrile was used by Morales-Cruz et al. (138) to reduce lysozyme aggregation during the encapsulation process in PLGA. This led to less enzyme inactivation, resulting in about 96% activity.

Lee et al. (139) conducted an *in vitro* study of lysozyme in poly(lactide-co-glycolide) (PLGA) microspheres. They added sucrose acetate isobutyrate (SAIB) to improve the encapsulation efficiency of lysozyme in PLGA. Then, to control the release of lysozyme from encapsulation, the team employed sulfobutyl ether β-cyclodextrin 7 sodium salt (Captisol®) (SBE-CD) which decreased the interactions between SAIB and PLGA. The amount of SBE-CD was varied in order to determine the optimal release and activity of lysozyme. The optimal microspheres exhibited the highest bioactivity retention for over 60 days at just below 95% activity against *M. luteus*.

Yu, L. et al. (75) encapsulated lysozyme in a PLGA-PEG-PLGA triblock copolymer. The copolymers were prepared via lactide and glycolide ring-opening polymerization reactions using PEG as the initiator. The polymer mix ratio was critical to the activity (75). The enzymatic activity of released lysozyme was slightly lower than that of free lysozyme after 45 days.

Lysozyme was encapsulated in a nanocomposite made of neutral polylactic acid (PLA) and PLGA in the work of Park et al. (140) who were interested in controlled protein release. They added organic solvent as it was thought to affect the release of encapsulated lysozyme. The researchers used an electrohydrodynamic jetting process in which electrostatic forces held the two polymers, PLA and PLGA, together. Different solvents were tested for optimizing lysozyme’s activity and stability and it was found that all particles had about a 5% bioactivity loss after 21 days of incubation using the *M. luteus* assay method. The authors suggested that the retention of activity was due to the optimized release rates of lysozyme as well as there was no significant alteration to the enzyme’s structure from the immobilization process.

Another example of lysozyme encapsulation was provided by Gaudana et al. (76) Encapsulation was performed using different ratios of a hydrophobic ion-pairing (HIP) complex to PLGA in the presence of dextran sulfate as a complexing agent. HIP complexes are formed through electrostatic interactions. The entrapment of lysozyme increased as the amount of PLGA increased in the preparation of the nanoparticles. Sustained release of lysozyme from the nanoparticles was observed for 28 days. The activity of lysozyme dissociated from the HIP complex did not show a significant difference as compared to the activity of free lysozyme. Although conformational changes may have occurred during the complexation process, after dissociation, lysozyme regained its natural conformation.

Another study by Reinhold et al. (141) showed that lysozyme could be loaded into PLGA microspheres through incubation. The authors modified the porosity of the pre-formed PLGA microspheres by incorporating various levels of pore-forming agent magnesium carbonate, MgCO₃. This group tested lysozyme enzymatic activity and observed full enzymatic activity was recovered after 28 days of release when MgCO₃ was used. It
was shown that when a higher concentration of MgCO₃ was used, higher activity was seen. Furthermore, it was concluded that the milder method employed for protecting the protein during encapsulation could also be attributed to the higher activity.

Swed et al. ([77]) encapsulated lysozyme in PLGA nanoparticles for biomedical applications using a novel phase separation method. A suspension of lysozyme in glycofurol and sodium chloride was obtained followed by the addition of the suspension to a PLGA solution. The suspension was mixed with ethanol which resulted in phase separation and formation of the desired, encapsulated lysozyme PLGA nanoparticles. The bioactivity of encapsulated lysozyme was 96% of free lysozyme activity using their process.

Tran et al. ([142]) encapsulated lysozyme in PLGA microparticles utilizing a carbon dioxide medium in a non-toxic isosorbide dimethyl ether solvent. Due to non-specific adsorption of lysozyme to PLGA along with ionic interactions, the lysozyme structure was partially denatured which caused some loss of bioactivity when tested against *M. luteus*. The amount of bioactivity loss was not specified but the majority of bioactivity was preserved.

Similarly, Ansary et al. ([143]) encapsulated lysozyme in double-walled PLGA microspheres in order to slow the release of lysozyme. PLGA was first dissolved in acetic acid and was then combined with a solution of lysozyme. Dichloromethane (DCM) was added and the microspheres were isolated by phase separation. This created the first layer of the microspheres. The microspheres were again treated with a solution of polyvinyl alcohol (PVA) forming the second layer of the double-walled microspheres. Evaporation of acetic acid and DCM hardened the resulting microspheres. The bioactivity of the double-walled microspheres was examined after 70 days. Lysozyme release was incomplete and activity had been preserved around 99%.

In another study performed by Lee et al. ([144]) a solid-in-water-in-oil-in-water (S/W/O/W) method was employed to stabilize lysozyme more effectively than a related water-in-oil-in-water (W/O/W) method during the encapsulation with PLGA. Similarly, SBE-CD was used again as the enzyme stabilizer. The interactions between the hydrophobic portions of lysozyme and cyclodextrin derivatives created enzyme shielding. Over the course of 30 days, the bioactivity of lysozyme remained above 80%.

Haji Mansor et al. ([145]) used a combination of PLGA and PEG to encapsulate lysozyme. The charge of lysozyme was neutralized by dissolution in different concentrations of sodium chloride (NaCl) solution which allowed for hydrophobic interactions between lysozyme and PLGA. Precipitated lysozyme material was then added to a polymer solution of PLGA-ester, PLGA-COOH and PEG-PLGA in dimethyl isosorbide (DMI) which provided encapsulated lysozyme. To preserve bioactivity, glycofurol was added to each enzyme solution prior to encapsulation which did not cause significant lysozyme denaturation. When the bioactivity was tested with *M. luteus*, it was preserved after 72 h.

Ma et al. ([78]) encapsulated lysozyme in poly(-caprolactone)--poly(ethylene glycol) copolymer block (PCL-b-PEG). Lysozyme was dissolved in a PCL-b-PEG solution and then mixed with a α-cyclodextrin (α-CD) solution. The concentrations of the two solutions were varied in order to optimize the conditions for preserving lysozyme activity. It was found that 2.0 wt% PCL-b-PEG/6.0 wt% α-CD resulted in the highest preservation of lysozyme activity over the course of 14 days. The resulting activity was around 80% which was higher than that of free lysozyme after 14 days at 36%.

Li, Y. et al. ([146]) used electrospinning to encapsulate lysozyme. Lysozyme-oleate complexes were formed to increase its solubility in an organic solvent combination of dimethyl sulfoxide (DMSO) and chloroform (CHCl₃). The dissolved lysozyme could then be loaded directly into poly-ε-caprolactone/polyethylene glycol, non-woven membranes. After 7 weeks, lysozyme’s activity was around 80%. The authors attributed the activity preservation to the formation of lysozyme-oleate complexes as well as enhanced stability from the PEG in the nanofibers.

Ali et al. ([147]) encapsulated lysozyme in an ammonia methacrylate copolymer (Eudragit RL), PLGA, and PEG-PLGA, using PEG as the preparation solvent. After each polymer was dissolved in PEG, lysozyme was dissolved in distilled water and added to the polymer-PEG solutions to form lysozyme-containing nanoparticles.

![Figure 13](image-url). Co-polymer nanoparticle formation ([147]). Reprinted from International Journal of Pharmaceutics, Vol 456. Mohamed Ehab Ali, Alf Lamrecht. Polyethylene glycol as an alternative polymer solvent for nanoparticle preparation. Copyright © 2013 with permission from Elsevier.
Bioactivity was fully preserved as a result of the encapsulation process. Moreover, lysozyme activity of the EDRL and PEG-PLGA nanoparticles showed no changes after storage for 30 days. The authors concluded that the stability may come from PEG’s protective effects on the enzyme through its specific binding with the enzyme.

Milacic et al. (148) also encapsulated lysozyme in PLGA and PEG. The polymers were assembled as cylindrical implants, or block polymers, in which lysozyme was encapsulated through a solvent extrusion method (149). Neutralization and pore-forming was completed using MgCO₃. As a result, lysozyme had few structural changes after 21 days and the majority of its activity was preserved.

Another example where PEG was employed to encapsulate lysozyme was in the work of Koda et al. (150) Lysozyme was first dissolved in an amphiphilic/fluorous PEGylated and perfluorinated random copolymer and water. The solution was dispersed by the subsequent addition of 2H,3H-perfluoropentane (Figure 14). Through this process, the secondary structure of lysozyme remained intact and 98% of lysozyme activity was preserved against M. luteus.

4.3.2. The immobilization of lysozyme on polyethylene glycol (PEG) via encapsulation

To this point, PEG has been discussed as a covalent linker between a solid support and lysozyme, and as a copolymer within an encapsulation formulation. It has also been used in other immobilization techniques for a variety of reasons. PEG has low toxicity, a good therapeutic window, and is non-destructive towards other polymers, and has miscibility in water (147). PEG has been attached to lysozyme for more efficient encapsulation. This is due to its ability to protect lysozyme from harsh microencapsulation conditions. Lysozyme’s release is also sustained over a longer period of time and enzyme aggregation is infrequent after PEGylation.

Diwan et al. (151) PEGylated lysozyme with methoxy polyethylene glycol that was encapsulated in PLGA. Lysozyme solution in borate buffer was treated with activated methoxy PEG succinimidyl succinate solution in a process that provided pegylated lysozyme. The acquired microspheres were then dissolved in acetonitrile and combined with pegylated lysozyme to achieve encapsulation. PEGylation was shown to be capable of protecting the protein from outside conditions that could denature it while minimizing the initial burst of protein for a sustained release of lysozyme in vitro. The results showed high retention (101.3 ± 10.4%) of specific activity of released lysozyme against M. luteus.

Ye et al. (152) developed a new hydrogel-immobilized lysozyme to inhibit both gram-negative E. coli and gram-positive B. subtilis bacteria growth in water. The porous hydrogel-immobilized lysozyme was prepared by polymerizing poly(ethylene glycol) methyl ether acrylate monomer via UV irradiation in the presence of the enzyme, thus immobilizing lysozyme into the matrix. The activity of the immobilized lysozyme was compared to that of the free lysozyme against Micrococcus lysodeikticus. They found that the immobilized lysozyme exhibited a relative activity that was 120–250% higher compared to that of lysozyme powder or free lysozyme in a water solution at room temperature. Furthermore, activity duration lasted up to 55 days, and the immobilized lysozyme demonstrated a wide temperature window and it was shown to have good recycling capabilities of up to 11 cycles with a high activity of 30% retained. It was observed that the hydrogel’s large surface area allows for higher lysozyme and bacteria interactions, which explains the higher activity. However, over time, those interactions decreased due to accumulating debris on the hydrogel surface.

4.3.3. The immobilization of lysozyme within other polymers via encapsulation

Whitaker et al. (153) studied the encapsulation of lysozyme in poly(D,L-lactic acid). The enzyme was loaded into the microparticles through enhanced mixing and spraying. Enhanced mixing eliminates the need of processing polymers in organic solvent or at high temperatures by instead combining them with supercritical fluid. The lysozyme containing microparticles showed activity around 92%.

Ben Amara et al. (154) encapsulated lysozyme in low methoxy (LM) pectin for application in food preservation. LM pectin is a gelling agent that is isolated from the peels of citrus fruit. Spray drying was used to create microcapsules that encapsulated lysozyme.
Lysozyme and LM pectin were stirred together in an imidazole-acetate buffer solution until properly hydrated to counter the dehydrating effects of spray drying. At a neutral pH, lysozyme was bound to LM pectin through electrostatic interactions. High and low concentrations of LM pectin during the spray drying process led to a decrease in lysozyme activity, whereas an intermediate concentration of LM pectin successfully preserved lysozyme’s antimicrobial activity against *M. luteus*.

Fakhraei Lahiji et al. (51) trapped lysozyme inside of three different biopolymers, namely hyaluronic acid (HA), polyvinylpyrrolidone (PVP) and carboxymethyl cellulose (CMC) in an attempt to deliver therapeutic drugs using a transdermal system. The trapped lysozyme-biopolymers were prepared by dissolving either HA, PVP, or CMC in a phosphate-buffered saline solution and then mixing with lysozyme. HA proved to be most efficient in maintaining lysozyme’s activity through a temperature range of 4°C to 25°C. Furthermore, CMC was the least effective encapsulating biopolymer for maintaining lysozyme’s activity. This could be due to the conformational changes of lysozyme from possible covalent interactions with CMC. Encapsulated lysozyme activity was as high as 99.8 ± 3.8% against *M. luteus*.

Bozkir et al. (47) encapsulated lysozyme in lipid-polymer hybrid nanoparticles. Lysozyme was dissolved in phosphate buffer solution and then combined with sodium dodecyl sulfate (SDS), which acted as an ion-pairing agent. The result was hydrophobic ion-pairing (HIP) complexes that were formed through electrostatic interactions. These complexes retained up to 88.7% of lysozyme activity.

Raheja et al. (155) encapsulated lysozyme in electrospun polycaprolactone (PCL) fibers. The focus of their work was to make an economical and functional apparatus (a spinneret) for synthesizing both hollow and core-shell nanofibers. In doing so, they demonstrated that the spinneret can be used for encapsulation of biomolecules like lysozyme. The authors generated lysozyme encapsulated in PCL fibers and tested the bioactivity of the fibers. The results showed that 95% of lysozyme activity towards *M. luteus* was preserved after encapsulation.

Ghassemi et al. (156) observed that lysozyme activity increased to ca. 118% after being encapsulated in poly (lactic-co-hydroxymethyl glycolic acid) (PLHMGGA) at the end of 60 days. They found that the secondary and tertiary structures of lysozyme were preserved. To obtain these results, it was found that the polymer concentration needed to be at 20% w/w.

Seif et al. (157) encapsulated lysozyme in a PVA solution to create fibers for therapeutic purposes. Acetic acid was added to facilitate the electrospinning process that uses electrostatic forces to create the fibers. These conditions were mild enough to preserve the lysozyme structure within the PVA. However, after the addition of glutaraldehyde, which lead to covalent bonding, lysozyme lost 50% of its activity due to its structure being partially denatured.

The degradable polyanion, poly(phthalic ethylene glycol ester), was used to encapsulate lysozyme layer-by-layer in the work of Hiraoka et al. (158) The two materials were bound together through ionic interactions after mixing in phosphate buffer solution. It was found that 75% of lysozyme activity was preserved in this method and the secondary and tertiary structures of lysozyme remained unchanged, similar to the observation of Ghassemi et al. (156).

The use of poly-L-Lactide (PLLA) porous microparticles to encapsulate lysozyme was studied by Kang et al. (159) The lysozyme encapsulation involved loading lysozyme into the pores of the polymer by mixing the two in a solution followed by precipitation using a compressed anti-solvent (160). When they tested with *M. luteus*, lysozyme activity was retained at 94.9% against the gram-positive bacteria (159). This activity is likely due to minor lysozyme conformational changes along with the precipitation with the polymer that could prevent lysozyme’s inactivation. Other reports by the authors showed that the activity of lysozyme improved with a better combination of active groups between lysozyme and substrate after solution enhanced dispersion by supercritical fluids (161). Slight loss in enzymatic activity could be due to the low efficacy of emulsifier PF-127 in preventing lysozyme unfolding at the water-methylene chloride interface and entrapment of LSZ in the PLLA layer (162).

### 4.4. Summary of Immobilization Techniques using polymers

In this section, we have reviewed research that has employed polymers in two different constructs, namely as the solid support and as a linker. The immobilization techniques utilized covalent bonding, adsorption and encapsulation. The resulting bioactivity of the immobilized lysozyme varied depending on the construct. Lysozyme that was covalently bonded to different polymers was shown, in some cases, to be effective towards both gram-positive and gram-negative bacteria. Typically, activity decreased after immobilization through covalent bonding. High activity preservation occurred with systems that had less steric hindrance.

Surfaces having chemical groups with steric bulk can create steric hindrance around lysozyme’s active site and it was found that this resulted in lower activity. In some cases, polymers were also employed as spacers between
the active site and the surfaces to minimize the effect of steric hindrance allowing for higher lysozyme activity.

One interesting observation was that the specific point used within lysozyme to attach the enzyme to the polymer impacted the resulting bioactivity of lysozyme. Sert et al. (126) reported that activity was 7.5 times higher when immobilization occurred at the amino-terminus rather than the carboxyl-terminus.

Another observation is that the polymeric solid support can possess activity and contribute to the overall antibacterial effect, similar to that observed for chitosan. For example, polymers with quaternary ammonium groups displayed their own antibacterial properties.

The reported studies by Yu, Q. et al. (115, 116) and Dias et al. (73) showed that complexes created by adsorption were typically more effective towards lysing gram-positive bacteria than gram-negative bacteria, as with covalent bonding. Nonetheless, this technique was not reported as often in the literature, likely due to a weaker interaction with lysozyme.

The general trend for encapsulation showed that there was little loss in lysozyme's activity over the course of 28–30 days. Encapsulation served as a form of protection of lysozyme from its environmental conditions. Preservation of activity was further improved with additives as seen in the work of Nam et al. (136), Pérez et al. (137) and Morales-Cruz et al. (138) These additives were shown to reduce lysozyme's conformational changes during the encapsulation process or to prevent lysozyme aggregation during the encapsulation process. Some cases demonstrated greater loss in activity, such as when lysozyme was encapsulated in fibers in Seif et al.'s (157) work due to covalent bonding that occurred during the encapsulation process.

5. Minerals

Some minerals have antimicrobial properties (163). By immobilizing lysozyme to minerals, antimicrobial properties can be enhanced for use in various industries, specifically for filtration purposes. These applications have been demonstrated for use in removing contaminants during wastewater treatment, purifying water, and optimizing food processing (163, 164). Lysozyme immobilized by minerals could also be used to improve medical treatments, for example, burn and wound treatment (165).

5.1. Covalent bonding to minerals

Kroll et al. (163) synthesized yttria stabilized zirconia (YSZ), a form of ceramic, into microtubes and immobilized lysozyme via a two-step process (Figure 15). The first step was hydroxylation of the microtubes, which could be done using either an acidic or an alkaline method. In their method, the acidic hydroxylation was performed using piranha solution, whereas the alkaline hydroxylation employed sodium hydroxide. The hydroxylation step was followed by silanization with 3-aminopropyl triethoxysilane to generate terminal amino groups on the membrane surface. Lysozyme was then covalently bound to the amino-activated membrane of the microtubes via an amidation reaction involving the carboxylic acid containing residues of lysozyme and the coupling reagent EDC. The antibacterial activity of the immobilized lysozyme was tested against *Micrococcus luteus* and showed that the total activity increased by a factor of 2 when lysozyme is covalently bound to YSZ as compared to lysozyme activity after unspecific binding. Comparisons to free lysozyme were not made.

Zhao, Q. et al. (164) immobilized lysozyme on halloysite nanotubes. Halloysite nanotubes are composed of aluminosilicate clay and exhibit antimicrobial properties of its own. Immobilization was achieved via covalent bonding by modifying the halloysite nanotubes to possess carboxylic groups followed by an amidation reaction involving the amino groups of lysozyme. The covalently bound lysozyme was then added to a polyethersulfone (PES) polymer solution to prepare hybrid antibacterial ultrafiltration membranes. The lysozyme-containing membranes exhibited 63% bacteriostatic activity against *E. coli*.

Mogilnaya et al. (165, 166) immobilized lysozyme on modified detonation nanodiamonds (MND) for application within the biomedical industry. Experiments were performed to analyze the difference between the antibacterial activity of lysozyme that was adsorbed to MND and lysozyme that was covalently bound to MND. To achieve adsorption, lysozyme and MND were mixed together in solution. The mechanism and specific type of absorption was not elucidated. Lysozyme was covalently bound to MND using benzoquinone as a linker in a multistep reaction process. The antibacterial activity of the covalently bound and adsorbed materials versus free lysozyme was determined using two gram-negative bacteria, *Photobacterium phosphoreum* (*P. phosphoreum*), and *E. coli* as well as one gram-positive, *B. subtilis*. There appeared to be higher antimicrobial activity for free lysozyme towards gram-negative *P. phosphoreum* and *E. coli* than for either the covalently bound or adsorbed lysozyme. Finally, the lysozyme-MND system was found to be also less effective towards gram-positive *B. subtilis* than free lysozyme. Nonetheless, in their studies, lysozyme
that was covalently bound to MND had higher activity than that of the lysozyme adsorbed to MND.

Li, H. et al. (79) immobilized lysozyme within a silica matrix through glutaraldehyde cross-linking. The silica matrix was amino-functionalized which reacted with glutaraldehyde. After cross-linking, the silica matrix was added to a lysozyme solution. It was observed that the lysozyme was bound to the surface of the silica beads, rather than inside the pores, which allowed lysozyme better access to bacteria. This method showed that bound lysozyme exhibited a 95.5% activity towards S. aureus and an 89.6% activity towards E. coli.

Crapisi et al. (64) immobilized lysozyme on non-porous glass beads through covalent attachment using glutaraldehyde as a linker. The lytic activity of the immobilized lysozyme was studied with M. luteus and compared to that of free lysozyme retained an activation yield (defined as percentage ratio of immobilized active enzyme to immobilized enzyme) of 12.8%. Furthermore, they found that the lytic activity diminished after 10 days and was 50% at day 4 relative to free enzyme.

5.2. Adsorption to minerals

Cheng et al. (167) performed research on the adsorption of lysozyme to zinc oxide (ZnO) and cerium oxide (CeO2) nanoparticles. Experimental conditions were at neutral pH (7.4), which is higher than the isoelectric point of both CeO2 and ZnO nanoparticles (ca. pH 3 or 3.5, respectively) and so both nanoparticles were negatively charged. As lysozyme has an isoelectric point (pI) at ca. pH 11, it was positively charged. This led to the conclusion that electrostatic interactions between the negatively charged nanoparticles and positively charged lysozyme are most likely the dominant interactions under the neutral pH condition (ca. pH 7.4). The activity for lysozyme adsorbed to Cerium (IV) oxide (CeO2) (86%) was slightly lower, as compared to the activity of lysozyme-zinc oxide (90%) against M. luteus. This is most likely due to their different isoelectric points (pI). The isoelectric point of ZnO is approximately at pH 3.5 which could have led to different binding sites compared to CeO2.

Yang et al. (168) studied the immobilization of lysozyme to layered double hydroxide nanocomposites through electrostatic forces. These nanocomposites were composed of magnesium aluminide (MgAl) and nitrate (NO3). The results indicated that lysozyme adsorbed to these nanocomposites had lower antimicrobial activity towards S. aureus than free lysozyme. However, the duration of the antibacterial effect of the lysozyme nanocomposites was held constant for a longer period of time compared to that of free lysozyme.

The work of Wang, Y. et al. (45) showed that when lysozyme was immobilized to halloysite nanotubes (HNT) through electrostatic interactions with a double hydroxide (an anionic clay), the lysozyme-HNT was able to lyse E. coli more effectively than free lysozyme (Figure 16).

Lu et al. (80) also adsorbed lysozyme to mesoporous silica and attributed the adsorption to electrostatic interactions. The silica was mixed with several different buffer solutions containing lysozyme at different pHs. It was found that a pH 10 was optimal for adsorption, similar to what was observed by Moerz et al. (169) The antibacterial activity was found to be 95% and higher.

Xiao, Q. et al. (170) immobilized lysozyme onto silica nanospheres and nanotubes. Adsorption was attributed to hydrogen bonding interactions between the Si–OH groups found on the surfaces of both the nanospheres and nanotubes and the amino and/or carboxylic groups of lysozyme (Figure 17). The lysozyme
immobilized on the nanotubes showed a higher activity than the lysozyme immobilized on the nanospheres. According to the authors, this is likely due to the airtight structure of the hollow nanospheres compared to the typical solid nanosphere structure. An airtight structure can negatively affect the diffusion process which involves the flow of substrates through the material.

Ding et al. (81) also immobilized lysozyme onto silica nanotubes. However, there was no discussion on the effect of the Si–OH groups, which were said to be responsible for hydrogen bonding between lysozyme and silica materials as discussed by Xiao Q. et al. (170) Instead, electrostatic interactions between negatively charged silica nanotubes and positively charged lysozyme was said to be responsible for lysozyme immobilization. It was found that immobilizing lysozyme at 150 mg/g of silica provided the highest antimicrobial activity at almost 90%.

Lee et al. (171) immobilized lysozyme onto extruded-shaped Na-Y zeolite in a recirculating packed bed reactor (RPBR). They studied the cell disruption of M. lysodeikticus using the immobilized lysozyme. Under the optimal conditions, the system achieved 93% cell disruption with 400 µg/ml protein content released from the bacteria. They also reported 12 successive cycles with the immobilized lysozyme.

Liu et al. (172) reported a ‘proof-of-concept’ for controlled immobilization of lysozyme into the interstitial spaces of a silica/organosilane superhydrophobic coating. They investigated the interfacial characteristics and surface chemistry of these coatings in depth as well as the antibacterial activity demonstrating that greater than 99.9% and 99.7% reduction in bacterial adhesion/growth for Salmonella Typhimurium LT2 and Listeria innocua on these surfaces, respectively. The authors report that the adsorption was due to electrostatic complexation between the lysozyme and the silica nanoparticles.

Perevedentseva et al. (173) performed electrostatic adsorption of lysozyme to carboxylated nanodiamonds (ND) of various sizes. Adsorption occurred via through electrostatic interactions between the ND’s negatively charged carboxyl groups and the positively charged amino groups of lysozyme. The formation of hydrogen bonds between lysozyme and ND’s was also thought to have occurred. Lysozyme that was immobilized to ND’s 100 nm in size showed the highest activity, roughly 70% of that of free lysozyme, against E. coli.

Vervald et al. (174) observed the enzymatic activity of lysozyme after bilayer adsorption on nanodiamonds (NDs) of various sizes in water. The lysozyme underwent more conformational changes in the first layer than the second, which led to the first layer showing 10–15% of free enzymatic activity while the second layer showed up to 60% of free enzymatic activity. Enzymatic activity was better retained with ND’s 100 nm in size.

Wang, Y. et al. (27) reported a new, facile, and oil-free approach to synthesizing mesoporous silica nanoparticles (MSNs) with controllable structure, specifically with tunable particle size (79–160 nm), as well as pore size. The group showed that these MSNs could absorb lysozyme and be implemented as antimicrobial enzyme delivery vehicles for antibacterial application. Their best results were demonstrated when the MSNs had a small particle size (79 nm) with large pores (22.2 nm). It was suggested that these results were due to the larger pore size of the MSN such that lysozyme was not only absorbed on the outer surface but within the cavity of the MSN, which would also result in protecting the enzyme from denaturing. Furthermore, these particles exhibited sustained release patterns of the lysozyme, approximately 67% released within 48 h. When compared to free lysozyme, their best lysozyme loaded MSN had the lowest MIC value of 500 µg mL⁻¹ when tested against E. coli, whereas free lysozyme exhibited limited inhibition towards E. coli even at the concentration of 1000 µg mL⁻¹. Finally, long-term antibacterial activity was also investigated and to their surprise the lysozyme loaded MSN maintained 100% inhibition towards E. coli for 5 days.

Figure 17. Immobilization of lysozyme from aqueous solution onto solid silica nanoparticles and hollow silica nanoparticles (80). Reprinted with permission from Elsevier. Chemical Engineering Journal, Vol 37. Qing-Gui Xiao, Xia Tao, Hai-Kui Zou, Jian-Feng Chen. Comparative study of solid silica nanoparticles and hollow silica nanoparticles for the immobilization of lysozyme. Page 40. Copyright © 2008 American Chemical Society.
Song, H. et al. (26) investigated the enhanced adhesion of silica nanopollens for long-term bacterial inhibition against *E. coli*. Modeled after a nature-inspired concept to mimic the pollen grain’s structure and adhesive properties, which easily adheres to the hairy legs of a honey bee, rough mesoporous silica nanopollens were designed to adhere to the ‘hairy’ bacterial surface and thus improve lysozyme delivery efficacy. The authors demonstrated a new synthesis for rough mesoporous silica hollow spheres (R-MSHS) through a one-pot, surfactant-free, cheap and scalable approach. Both smooth (S-SHS) and rough silica nanopollens were synthesized with a mean particle diameter measured to be $256 \pm 17$ nm. The R-MSHS demonstrated better and stronger particle-bacteria adhesion than that of the S-SHS. Furthermore, they showed that both silica nanospheres possess relatively low toxicity towards *E. coli* themselves, however, the R-MSHS exhibited higher toxicity over the S-SHS due to the stronger interaction with the bacteria membrane. Upon loading lysozyme onto the silica nanopollens through electrostatic forces, it was shown that the rough surface allowed for a higher loading than that for the smooth surface, as well as, a higher antibacterial activity and a prolonged bacterial inhibition up to 3 days towards *E. coli*.

In the study by Xu, C. et al. (25) novel rod shaped hollow mesoporous silica nanoparticles (HMSNs) with large and small cone shaped pores, HMSN-LP, 40 nm and HMSN-SP, 3 nm, respectively, were synthesized using an oil–water system. Lysozyme was loaded onto the HMSNs and the systems’ antimicrobial activity were tested against an *E. coli* biofilm model using a LIVE/DEAD bacterial viability kit. Using this method, the group was able to show that the lysozyme loaded HMSN-LP had the highest antimicrobial activity compared to HMSN-SP and free lysozyme. Furthermore, their data indicated that the HMSN-LP mainly diffused into the extracellular polymeric substance of the biofilm but not inside the bacteria.

### 5.3. Encapsulation within minerals

Shi et al. (48) doped porous calcium carbonate (CaCO$_3$) with heparin to encapsulate lysozyme. This technique retained almost 100% of lysozyme’s antibacterial activity against *M. luteus* relative to free lysozyme. According to the researchers, this was due to a high enzyme loading capacity with optimal particle shape. Particle shape influences loading capacity and protein morphology (175). In their study, lysozyme was loaded into CaCO$_3$ pores through electrostatic interactions by doping the CaCO$_3$ with polyanionic heparin to induce an overall negative charge. A negative charge increased lysozyme’s ability to be adsorbed (Figure 18). Encapsulation was achieved by creating polyelectrolyte multilayers through layer-by-layer assembly (48).

Shi et al. (176) continued the previous work and further described the attached lysozyme to heparin-doped CaCO$_3$ microparticles via adsorption through electrostatic interactions and secondary hydrophobic interactions. The activity of released lysozyme activity was measured and it was observed that after 3 cycles of the reload-release of lysozyme, lysozyme maintained an activity through each cycle of around 110%. Free lysozyme, on the other hand, showed lower activity around 100%.

Hassani et al. (82) used supercritical CO$_2$ to encapsulate lysozyme in CaCO$_3$ microparticles. To load the microparticles, lysozyme was first dissolved in an aqueous calcium carbonate solution. The solution was stirred as supercritical CO$_2$ was added. The encapsulated lysozyme retained almost 100% of free lysozyme activity.

![Figure 18](image-url) (a) synthesis of the Hep/CaCO$_3$ particles, (b) loading the lysozyme into the Hep/CaCO$_3$ particles, (c) consecutive adsorption of oppositely charged polyelectrolytes by the layer-by-layer method on the protein loaded template, (d) sacrificing the template to generate polyelectrolyte capsules with the encapsulated lysozyme (48). Reproduced with the permission of Royal Society of Chemistry.
5.4. Summary of immobilization techniques using minerals

The work reviewed in this section show that positive antibacterial results can be achieved when lysozyme is immobilized to different minerals. As seen in other sections for the immobilization of lysozyme through covalent bonding, activity was typically reduced compared to free lysozyme. However, Li, H. et al. (79) demonstrated an activity increase of lysozyme when covalently bound to a matrix surface rather than within it because the lysozyme was better exposed to bacteria.

Adsorption trends showed a high retention of lysozyme activity compared to free lysozyme. It was discovered that some minerals provided lysozyme with a higher antibacterial activity than other minerals based on their isoelectric points during adsorption. This was most likely due to the fact that isoelectric points determined the binding sites where adsorption would take place. Furthermore, electrostatic interaction promoted a higher activity as well as its antibacterial duration in solution against gram-negative bacteria. Wang, Y. et al. (45) showed that adsorption of lysozyme to an anionic material improves the activity of lysozyme towards gram-negative bacteria.

Encapsulation of lysozyme within minerals showed that lysozyme activity was preserved almost completely compared to free lysozyme. This was due to high encapsulation efficiencies with little conformational changes to the lysozyme.

6. Metals

Immobilizing lysozyme to metals has been studied intensely for medical device applications. The intent of this research is to lower the risk of infections from surgical tools. Some metals, such as silver, possess their own antimicrobial properties. In the literature, there are reports of other non-antimicrobial metals pretreated with cross-linkers to assist with immobilization of lysozyme.

6.1. Covalent bonding to metals

Minier et al. (177) covalently bound lysozyme to a stainless steel surface to target medical device and food-processing industries. The metal oxide surface of stainless steel was primed with amino(propyl)triethoxysilane self-assembled monolayers that were crosslinked via an imine linkage to glutaraldehyde, a convenient molecule to use to cross-link proteins. The terminal aldehyde of the glutaraldehyde was then coupled with an amine group of lysine on lysozyme to give covalently bound the enzyme (Figure 19). The covalently bound lysozyme to a stainless steel surface via a glutaraldehyde linker displayed significant lytic activity whereas little activity was observed for derivatives without the glutaraldehyde linker. However, there was loss of activity upon immobilization since it was thought the lysine residues involved in the immobilization reduced access to the active site from the steric hindrance.

Wu, J. et al. (83) covalently immobilized lysozyme on superparamagnetic beads. The authors employed their PRECISE system (Protein Residue-Explicit Covalent Immobilization for Stability Enhancement) to immobilize the lysozyme on the beads. The technique is site-specific and utilizes arginine, lysine and N-terminus groups found within lysozyme. The enzymatic activity of immobilized lysozyme against E. coli demonstrated a loss of about 50% of activity as compared to free lysozyme due to steric hindrance.

Liburdi et al. (178) covalently immobilized lysozyme to magnetic (iron) non-porous polystyrene beads containing two different activation groups, namely, tosyl-activated beads (TSA) and carboxylated beads (CA) in order to generate an anti-microbial agent for use in the wine industry. For the tosyl-activated beads, a secondary amine linkage was created via a substitution reaction of the tosyl group. For the carboxylated beads, EDC was used as a coupling agent to couple acid moieties on the bead with amine groups on lysozyme via amidation reactions. The order of highest enzymatic activity to lowest against gram-positive Oenococcus oeni was LYS-TSA, LYS-CA and free LYS.

Ahmady et al. (179) synthesized lysozyme bioconjugates by reducing aryldiazonium gold(III) salts ([HOOC-≡C₆H₄N≡N]AuCl₄) with lysozyme to form benzoic acid gold nanoparticles (AuNP-COOH). The hydrolytic activity of the lysozyme bioconjugate was measured using a turbidimetric assay with M. lysodeikticus and they found a 68% increase in the hydrolytic activity of the enzyme bioconjugate compared to that of the native lysozyme in the first minute. Furthermore, the bactericidal activity of the AuNP-lysozyme bioconjugate was studied against a selection of gram-negative and gram-positive standard ATCC strains (E. coli, K. pneumonia, S. typhimurium, and S. aureus) as well as clinical resistant isolates: beta lactamase producing E. coli and imipenem-resistant P. aeruginosa. The antibacterial activity was enhanced by 98–99% compared to that of the native lysozyme.

6.2. Adsorption to metals

Eby et al. (180) researched an antimicrobial coating that was created with lysozyme and silver nanoparticles. The
coating employed two different biocidal mechanisms which were the antimicrobial activity of the silver ions and the muramidase activity of lysozyme. To allow for the adsorption of lysozyme and silver onto a stainless steel blade, the blade was acid-etched and then submerged in a lysozyme--silver nanoparticle deposition solution. The blade coatings exhibited antimicrobial activity against a range of bacterial species and the silver nanoparticles contributed to most of the antimicrobial effects.

In another similar study, Wang, G. et al. (181) observed that adsorption of lysozyme onto silver nanoparticles occurred from hydrophobic interactions (Figure 20). This caused an alteration of lysozyme’s secondary structure, leading to lower bioactivity. Both Eby (180) and Wang (181) tested against gram-positive and gram-negative bacteria and Wang et al. observed roughly 3 log reduction of lysozyme activity between 1.5 and 3 h.

Ernest et al. (182) studied lysozyme adsorption onto silver nanoparticles that was mediated by lysozyme thiol interactions with the nanoparticles. The modified nanoparticles had a synergistic antibacterial activity towards E. coli.

Rösch et al. (84) adsorbed lysozyme onto titanium in the presence of albumin. Lysozyme was first immobilized to albumin through electrostatic interactions followed by adsorption onto the titanium. The negatively
charged titanium allowed for electrostatic interactions during adsorption. It was found that there was an increase of lysozyme activity in the presence of albumin. Kanjanakawinkul et al. (183) adsorbed lysozyme onto magnesium aluminum silicate (MAS) nanoparticles via hydrogen bonding and electrostatic forces. These interactions led to changes in lysozyme’s tertiary structure, which lowered its bioactivity when tested against M. luteus.

Chaudhary et al. (184) immobilized gold nanoparticles (Au(0) NPs) over the surface of nitrogen-doped, reduced graphene oxide (NrGO). The NrGO was synthesized via a hydrothermal treatment of graphene oxide (GO) in the presence of urea. Spectroscopic studies revealed a conformational change in lysozyme (Lys) and electrostatic interaction between Lys and Au(0)-NrGO. The Au(0)-NrGO-induced conformational changes in the structure of the enzyme resulted in a significant decrease in its activity at a certain concentration of Au(0)-NrGO.

Orhan et al. (185) investigated the immobilization of lysozyme through adsorption on magnetic poly(2-hydroxyethyl methacrylate (HEMA)-glycidyl methacrylate (GMA)) nanoparticles for potential usage in bacteria killing studies. The authors explored the activity of the lysozyme immobilized magnetic nanoparticles against Micrococcus lysodeikticus bacteria and demonstrated that all bacteria were destroyed within minutes.

Sonu et al. (186) compared the binding behavior and antibacterial activity of free lysozyme with its non-covalent assembly with silver (Ag) and gold (Au) colloidal nanoparticles (NPs) in presence of two drugs, namely, sulfadiazine and caffeine. The antibacterial activity of lysozyme in presence of the drugs showed a 9–14% upsurge with AuNP, in sharp contrast to ca. 31–34% decrease in AgNP.

6.3. Summary of immobilization techniques using metals

Covalent bonding of lysozyme to metals through a variety of cross-linkers lowered enzymatic activity due to steric hindrance around the active site. However, evidence has shown that lysozyme activity stability can be increased.

Silver, used for lysozyme adsorption, demonstrated its own antimicrobial activity towards both gram-positive bacteria and gram-negative bacteria and therefore synergistically contributed to the overall antimicrobial effect of the adsorbed lysozyme.

Adsorption proved to be more successful than covalent bonding approaches and this was hypothesized to be due to less steric hindrance in the system. However, hydrogen bonding weakened lysozyme’s activity more so than electrostatic interactions and it is hypothesized that the hydrogen bonding created harsher conformational changes in the lysozyme structure. Nonetheless, it was shown that adsorption through electrostatic interactions on these different metals did change the secondary and tertiary structure of lysozyme which lowered its bioactivity.

7. Graphene-based materials

Graphene is a material made up of solely carbon rings joined together. In the past graphene-based materials have been studied and applied in many different industries such as analysis, catalysis, biomedicine, environment, energy and electronics. The success of immobilizing lysozyme onto graphene-based materials and its ability to retain its bioactivity is correlated with the degree of oxidation of the graphene. A lower degree of oxidation has less impact on lysozyme’s conformation and function, which allows for a better biocompatibility. Adsorption of lysozyme onto graphene could be a result of π-π interaction, hydrophobic interaction, hydrogen bond, van der Waals interaction, and electrostatic interaction (187). The benefits of using these materials come from their chemical and physical properties, such as high surface to volume ratio, biocompatibility and electronic and mechanical capabilities. However, one challenge of using these materials is overcoming biosafety issues related to graphene within a biological system. After graphene enters a biological system, protein adsorption can occur immediately which can cause changes to the surface properties of graphene and the biological properties of the enzyme (3, 187).

Carbon nanotubes (CNTs) made of graphene are being used as tools in nanomedicine for diagnosis and...
therapeutic purposes. There are two types of CNTs, multi-walled and single-walled. Single-Walled Carbon Nanotubes (SWCNT) are known to have antimicrobial activity against gram-negative bacteria. They have the potential to be incorporated into fibers, polymer nanocomposites, electrical thin films, bio-inspired materials and have biotechnological applications \((86, 188–191)\). Multi-walled CNTs (MWCNTs) have a similar structure to SWCNTs but have additional layers of graphene. They are known for having a higher starting purity than SWCNT, are readily available and are low cost. \((42, 85)\).

7.1. Covalent bonding to graphene materials

Merli et al. \((85)\) covalently bound lysozyme to multi-walled carbon nanotubes (MWCNT). MWCNTs were first oxidized by acidic etching to create accessible carboxyl groups at the nanotube tips. Lysozyme was bound to the oxidized MWCNTs using EDC and NHS as coupling agents. Covalently bound lysozyme to MWCNTs exhibited higher antimicrobial activity towards \(S.\) aureus than free lysozyme in solution.

Puentes-Camacho et al. \((42)\) compared covalent and adsorption methods of lysozyme attachment to MWCNTs. For the adsorption method, Van der Waals, hydrogen bonding, electrostatic attraction, \(\pi \cdots \pi\) and hydrophobic interactions may have all aided in adsorption. For covalent binding, MWCNTs were primed with \(N\)-(3-dimethylaminopropyl)-\(N\)'-ethyl carbodiimide hydrochloride (EDAC) to produce an activated ester in the presence of NHS and 2-(N-morpholino)ethanesulfonic acid (MES) buffer. The activated ester was then coupled to lysozyme via a covalent amidation reaction. Although lysozyme covalently bound to MWCNTs had higher enzymatic activity than lysozyme adsorbed to MWCNTs, both systems had lower enzymatic activity than that of free lysozyme against \(M.\) luteus after 50 days. Adsorption showed a lower activity most likely due to lower stability of the complex relative to the covalent bonding method. Both methods showed a lower activity than free lysozyme \((42)\).

7.2. Adsorption to graphene materials

7.2.1. The immobilization of lysozyme on graphene oxide and reduced graphene oxide through adsorption (physical immobilization)

Duan et al. \((192)\) immobilized lysozyme with graphene oxide and chemically reduced graphene oxide on a poly-ethersulfone matrix. Immobilization was achieved via electrostatic and hydrophobic interactions for both lysozyme-containing composites (Figure 21). Plating with \(E.\) coli showed that the chemically reduced graphene oxide possessed a higher antimicrobial activity.

7.2.2. The immobilization of lysozyme on single wall carbon nanotubes through adsorption (physical immobilization)

Horn et al. \((86)\) demonstrated that lysozyme adsorbs to SWCNT through hydrophobic interactions involving lysozyme’s tryptophan groups. When hydrophobic interactions take place, \(\pi \cdots \pi\) stacking occurs, which potentially strengthens the adsorption. The lysozyme-SWCNT systems possesses 99% of free lysozyme’s activity against \(M.\) luteus and \(S.\) aureus. It was shown that the activity is maintained in the fibers, however, the authors did not report the duration of the activity.

Another example was reported by Horn et al. \((188)\) in which the cationic surfactant tetradecyl trimethylammonium bromide (TTAB) was incorporated into a lysozyme antimicrobial system. The goal was to use TTAB to increase demixing, which is when substances in a mixture separate, and increase SWCNT fiber strength.

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Figure 21. Immobilization principle of lysozyme on graphene oxide and chemically reduced graphene oxide \((192)\). Reprinted from Applied Surface Science, Vol 355. Linlin Duan, Yuanming Wang, Yatao Zhang, Jindun Liu, Graphene immobilized enzyme/polyether-sulfone mixed matrix membrane: Enhanced antibacterial, permeable and mechanical properties. Page 438. Copyright © 2015 with permission from Elsevier.
However, antimicrobial activity was 70% compared to that of free lysozyme.

Neupane et al. (193) used zeolitic immobilized frameworks (ZIF) and a one-pot synthesis to anchor large-substrate enzymes (lysozyme and α-amylase) onto carbon nanotubes (CNT) for biocatalysis. Their objective was to further understand the structure–function relationship of the immobilized enzyme to guide the rational design of future CNT-ZIF composites and to improve stability, loading capacity and catalytic efficiency of the immobilized enzymes. Lysozyme (T4L mutants) was adsorbed on the CNT-ZIF composite through electrostatic interactions, π–π interactions, and hydrophobic interactions. They demonstrated the activity of the enzyme using the commercial lysozyme activity kit (with Micrococcus lysodeikticus). Compared to the free lysozyme they showed that the CNT-lysozyme-ZIF complex retained activity.

7.3. Summary of immobilization techniques using graphene materials

Graphene materials have been shown to possess antibacterial properties towards gram-negative bacteria. In this section, there were some cases where immobilizing lysozyme to graphene materials showed enhanced activity and stability relative to free lysozyme. Merli et al. (85) demonstrated a higher activity for covalently bound lysozyme to MWCNTs, however, not all cases demonstrated the same effect. Puentes-Camacho et al. (42) observed a decrease in lysozyme activity when lysozyme was covalently bound to MWCNTs; however, their methods for covalent bonding did differ and is most likely the cause of the discrepancy.

Adsorption of lysozyme to graphene materials showed high preservation of lysozyme activity. This was most likely due to fewer conformational changes compared to covalent bonding and fewer adsorption interactions taking place at once.

Despite the advantages of using graphene materials, they may have a risk in posing toxicity to environmental and human health (194).

8. Alginate

Alginate is a natural heteropolysaccharide hydrogel of β-D-mannuronate and α-L-guluronate. It is commonly used as a gel, which can be formed through cross-linking with divalent ions. Using alginate as a support for lysozyme immobilization is beneficial due to its mild cross-linking conditions which do not require solvents that can negatively impact lysozyme’s antibacterial activity. Alginate has good biocompatibility, low toxicity, biodegradability and regenerability. Lysozyme-alginate complexes can be applied to the pharmaceutical industry for drug delivery or any other industry dealing with biological systems. Past research has only focused on this material being used for encapsulation of lysozyme (39, 54, 103).

8.1. Encapsulation within alginate materials

Zhang, J. et al. (103) studied rectorite-encapsulated lysozyme alginate nanogels. The authors generated cellulose nanofibrous mats and studied their antibacterial properties (Figure 22). The system demonstrated antimicrobial activity towards both E. coli and S. aureus with greater effects towards S. aureus. Specific interactions between the lysozyme and the material were not discussed.

Wells et al. (39) placed calcium alginate microspheres in a saturated lysozyme solution containing a low concentration of sodium chloride. The sodium chloride partially degraded the alginate and allowed for lysozyme uptake into the microspheres that were created through this process. Testing against M. luteus showed that lysozyme bioactivity was greater than 100% over a maximum of a 16 d period in some cases.

Matouskova et al. (195) studied the encapsulation of selected plant and animal antimicrobial substances (herbs, spices, lysozyme and nisin). Substances were packaged into liposomes and polysaccharide particles (alginate, chitosan and starch) and antimicrobial activity was tested against two gram-positive bacteria, Bacillus subtilis and Micrococcus luteus, and against two gram-negative strains, Escherichia coli and Serratia marcescens. Liposomes that were encapsulated with herb and spice...
extracts exhibited very good inhibitory effect against all
tested bacterial strains. The authors state that these anti-
bacterial materials could be used in the production of
food and food supplements, pharmaceutical and cos-
metic industries.

Jin et al. (54) created an electrochemically stimulated
alginate thin–film matrix for the entrapment of protein
drugs. Using the different interactions of Fe$^{3+}$ and Fe$^{2+}$
ions on an alginate matrix they were able to reversibly
form a thin–film hydrogel through cross-linking of the
Fe$^{3+}$ ions with the alginate on the electrode plate.
When this was performed in the presence of lysozyme,
the protein was physically entrapped within the film.
The lysozyme could then be released upon electroche-
mically reducing Fe$^{3+}$ to Fe$^{2+}$ as the interaction with
the reduced cation only weakly interacted with the algini-
tate, which resulted in the disintegration of the alginate
hydrogel. The results of this research showed that the
lysozyme demonstrated remarkable stability, robust-
ness, and activity after the applied potential and after
storage. The entrapped lysozyme after release demon-
strated ca. 74% activity compared to that of the lyso-
zyme in the bottle towards lysing M. luteus.

Ward et al. (196) reported the use of sodium alginate
as a potential ligand for enhanced colloidal liquid
aphrons (CLAs) immobilization. The authors state that
sodium alginate has been used in drug delivery appli-
cations due to its low toxicity and charged interactions
that allow for encapsulation. They used five model pro-
teins including lysozyme, with activity and conformation
comparable to their native counterparts. The use of pro-
teolysis showed that as the degree of ionic bonding
increased between the protein and sodium alginate,
the degree of protease resistance decreased due to con-
formational changes experienced during binding.

8.2. Summary of immobilization techniques using
alginate

In this section, lysozyme encapsulated in alginate
materials demonstrated higher activity towards gram-
positive than gram-negative bacteria. Mild cross-linking
conditions allow high preservation of lysozyme activity
during the encapsulation process. Zhang, J. et al. (103)
showed that rectorite-encapsulated lysozyme alginate
nanogels were effective towards both gram-positive
and gram-negative bacteria, with a higher activity
towards gram-positive bacteria. Another interesting
observation made by Wells et al. (39) showed that
soaking calcium alginate in sodium chloride partially
derogated the calcium alginate to allow for higher lyso-
zyme uptake. This allowed for a higher lysozyme activity
than that of free lysozyme.

9. Other

9.1. The immobilization of lysozyme within fabric
via covalent bonding

Wang, Q. et al. (87) created novel antimicrobial textiles.
Pre-treated wool was oscillated in a solution of glutaral-
dehyde. The wool was then rinsed with deionized water
and lysozyme was immobilized on the wool via covalent
cross-linking in a lysozyme buffer. The researchers
observed that 43% of the antibacterial activity of lyso-
zyme towards S. aureus was conserved after five cycles
of use.

9.2. The immobilization of lysozyme on a double
hydroxide via adsorption

Van der Waals attractions occur through hydrophobic
lysozyme amino acid and the double hydroxide.
Bouaziz et al. (11) demonstrated the adsorbed lysozyme
showed a lower antimicrobial effect towards
S. epidermidis than free lysozyme over the course of
24 h. Lysozyme adsorbed to lactate dehydrogenase
(LDH) seemed to have gone through a modification of
a few tryptophan groups.

9.3. Summary of immobilization techniques using
other materials

In this section, lysozyme had a lower activity than that of
free lysozyme when covalently bound to wool and
adsorbed to anionic clay known as a double hydroxide.
However, lysozyme covalently bound to wool showed
it could be recycled as it went through five cycles of
use and experienced some activity preservation.

10. Conclusion

Overall, research has shown that the immobilization of
lysozyme can increase the stability and lifespan of the
enzyme, and increase the activity of these hybrid
systems. Furthermore, immobilization of lysozyme
improves the ability of lysozyme to target not only
gram-positive bacteria but also gram-negative bacteria.
Some solid supports immobilizing lysozyme possess
their own antimicrobial or antibacterial properties or
could be manipulated to possess functional groups,
such as quaternary ammoniums, that have antimicro-
bial/ antibacterial effects. However, some antibacterial/
antimicrobial materials, such as graphene and graphene
oxide, have environmental and human health toxicity
concerns.
The activity of lysozyme can be altered depending on which functional groups of its amino acids are interacting with the solid support. These changes in activity typically happen when there are conformational changes or steric hindrance. For example, while covalent bonding is the strongest form of immobilization for lysozyme, the activity of the lysozyme tends to be reduced due to the physical changes within the structure of the enzyme caused by this type of interaction. In contrast, adsorption involves weaker surface interactions with lysozyme, and so it tends to disrupt the structure of lysozyme the least, and this technique provided comparable activity to that of free lysozyme.

Both covalent bonding and adsorption have shown fewer conformational changes when lysozyme was attached using spacers between lysozyme and the support surface. The distance created between lysozyme and that surface allowed for less steric hindrance, resulting in better antibacterial activity.

Encapsulation has been used to protect lysozyme from denaturation from its surrounding environment in order to extend its half-life while allowing release into its environment over time. However, the lysis ability is not improved in these methods. Some methods of covalent bonding and adsorption show similar activity preservation and stability compared to encapsulation. In each form of immobilization, lysozyme tends to exhibit higher activity against gram-positive bacteria over gram-negative bacteria; however, in a few cases, a higher lysis effects towards gram-negative bacteria have occurred.

One challenge with conducting a comparative analysis of the various research studies is that the findings of lysozyme activity is varying parameters. Oftentimes, lysozyme’s activity is reported as a percentage based on absorbance values of the lysozyme. There are other papers, such as one written by Song, Y. et al. (40) that measured lysozyme’s lysing success by percentage of bacteria cell lysis. Another variation of reporting lysozyme activity was providing the actual absorbance value of lysozyme without the conversion of that value into a percentage of activity as seen in the work of Edwards et al. (66) While other groups had opted for the LIVE/DEAD bacterial viability kit using dyes without quantifying the new bioactivity of the system. To advance the field of antibacterial/ antimicrobial studies with lysozyme on solid-supports, there should be further studies of such materials using standardized parameters to allow comparison of antimicrobial activities. The authors of the work reviewed in this paper had different research objectives and not all were interested on the antibacterial activity per se. Understandably, their tests and readouts were not harmonized.

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Additional notes
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