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The MIT Marine Industry Collegium
Opportunity Brief #43

Biotechnology and the Sea: Recent Advances and Applications

A Project of
The Sea Grant College Program
Massachusetts Institute of Technology
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BIOTECHNOLOGY AND THE SEA: RECENT ADVANCES AND APPLICATIONS

Opportunity Brief #43

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Marine Industry Advisory Services
MIT Sea Grant Program

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1.0 INTRODUCTION AND BUSINESS PERSPECTIVE

Recent advances in biotechnology have suggested new roles for marine materials and new solutions for marine problems. Derivatives from seaweed, shark, crustaceans and other marine biomaterials are finding their way into the food and pharmaceutical industries. The science of biotechnology will also increase our understanding of the aquatic environment. This Opportunity Brief reviews the results of several MIT research projects which highlight the link between biotechnology and the sea, including the isolation of a tumor growth inhibitor from shark cartilage, a DNA-hybridization probe to detect microorganisms in seawater, the use of marine biopolymers for controlled release of pharmaceuticals and food preservatives, and the prospects for commercial production and application of chitins and chitosans.

Ten years ago calf cartilage was found to contain a component which inhibited the growth of blood vessels towards tumors, and thereby slowed the growth of the tumors themselves. Research aimed at identifying this substance was severely restricted by the limited supply of calf cartilage and the low yield of inhibitor. Because the shark endoskeleton is composed entirely of cartilage and because that fish rarely has tumors, shark cartilage was examined as a possible source of the angiogenesis inhibitor. Experiments revealed that the inhibitor was present in a highly concentrated form. The combined effects of high concentration, high percentage of cartilage in shark, and increased animal weight produce an inhibitor yield that is 100,000 times greater from sharks than from calves, when measured on a per-animal basis. Research in progress focuses on identifying the specific entity responsible for inhibiting neovascularization, and on developing an in vitro assay to expedite the identification and purification of this factor.

By using DNA probe technologies, an MIT research project is developing a novel assay for detecting the presence of specific microorganisms in seawater. The DNA-hybridization technique, which depends upon the unique chromosomal signature of the target organism, can provide organism counts quickly and efficiently. Unlike traditional methods for assaying bacterial populations in water samples, this process yields a direct count of a specific microorganism, and is well suited for automated batch processing. The commercial success of this technique as an identifier of organic contaminants for the food industry suggests that it will quickly find its place in marine applications.

Several marine biopolymers show particular promise in controlled release technologies. The availability, low cost, and chemical and toxicological properties of alginates, carageenans, chitosans, and agar-derived polymers make these marine materials particularly well suited as controlled-release delivery devices. The encapusulation process is gentle, and complex molecules can be incorporated without denaturing the active agent. By directly controlling the release and diffusion of an encapsulated compound, improved results can be obtained with lower total delivery, and thus at lower cost. Drug blood levels can be accurately maintained over prolonged periods, without the "peak-and-valley" problems associated with conventional, discrete dosage schedules. By controlling the diffusion of preservatives in foods, high concentrations can be maintained at the food surface without significantly changing the bulk properties of the product. This technology has direct applications in food production and preservation, pharmaceuticals, pesticides, and wherever controlled release of an active agent is important.

Sea Grant sponsorship of chitin research was originally motivated by the need to find a use for the thousands of tons of shellfish waste dumped overboard annually. The research produced a wealth of information on the nature of chitin and chitosan, and identified many potential applications and processing techniques, as well as alternative, land-based sources of the raw material. Ray Pariser of the MIT Sea Grant College Program is completing his work as editor of The Chitin Sourcebook, a comprehensive review of chitin research to date. He hopes to see the results of university labors successfully transferred to commercial concerns, and has thoroughly investigated the commercial potential of chitin-based products and processes, product quality, production methods, existing patents, and the barriers to entry, both real and perceived. He concludes that the combination of an abundant, underutilized resource, a pool of important and known applications, and a growing understanding of the required processing methodology justifies a strong effort to overcome the few remaining obstacles to profitable commercial exploitation.

2.0 ISOLATION OF ANGIOGENESIS INHIBITOR FROM SHARKS

For many years scientists hypothesized that certain substances could inhibit the growth of new blood vessels. If additional blood vessels could be prevented from moving toward solid tumors, the tumors could not continue to develop. Because nearly all solid tumors require new blood vessels to grow beyond a millimeter in size, validating this hypothesis was a fundamental step to establish a foundation for a potential powerful new form of cancer therapy.

Cartilage from calves' scapulas contains a substance that inhibits the vascularization of solid tumors. This mammalian cartilage yielded the first inhibitor of neovascularization to be isolated, says Professor Robert Langer of the MIT Department of Applied Biology. No toxic effects were observed when this substance was infused into rabbits or mice, yet the growth of new blood vessels toward two types of implanted tumors ceased and tumor growth stopped. The single factor most limiting the further study of this substance is a shortage of supply. Mammals have only small quantities of cartilage, and after elaborate processing it takes 500 g of cartilage to produce 1 mg of partially purified inhibitor.

"It occurred to us that sharks could be a potential source of this inhibitor because, unlike mammals, sharks have an endoskeleton composed entirely of cartilage rather than bone," says Professor Langer. Cartilage comprises about 6% of the shark's total body weight, compared to less than 0.6% in calves. (See Figure 1.) In addition, since shark cartilage does not undergo the conventional developmental process of mineralization and neovascularization during bone formation, he wondered whether this cartilage could have a high concentration of a neovascularization inhibitor.

With help from MIT Sea Grant, Professor Langer acquired 20-ft-long basking sharks weighing 900 lbs from Fresh Water Company of Boston. After processing and purification, the yield of angiogenesis inhibitor was about a thousand times higher than in calf cartilage, as only 0.5 g of shark cartilage was needed to produce 1 mg of extract.

Three different tests were conducted on rabbits with the extract of basking shark cartilage, and in every case it significantly inhibited tumor neovascularization. After 19 days all corneas of rabbits not receiving the extract developed large, three-dimensional tumors with an average maximum blood vessel length of half the diameter of the cornea. In contrast, none of the corneas treated with shark cartilage extract grew three-dimensional tumors and the average maximum vessel length was 75% shorter than the controls. (The calf cartilage extract reduced the vascular growth rate by 70%.) (See Figure 2.)

"This result is noteworthy since it took 1000 times more calf cartilage to produce a substance that could cause an equivalent inhibitory effect," says Professor Langer. Because the tumor cells do not die in the presence of the inhibitor, the inhibitor appears to act on capillary advancement rather than on the tumor itself.

Figure 1. Shaded areas show extent of shark cartilage.

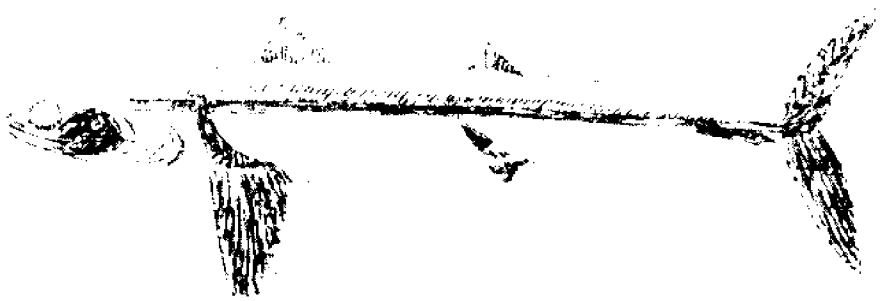
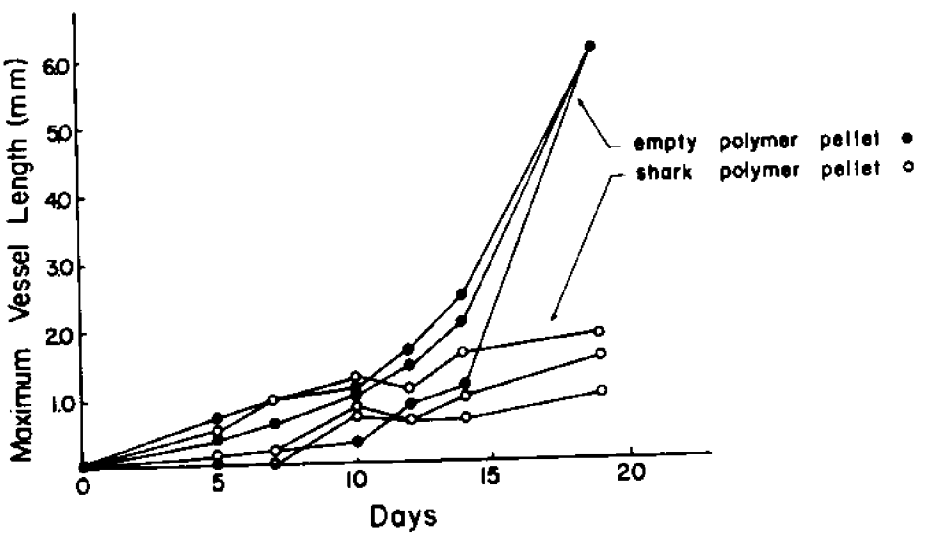


Figure 2. Pellet with shark polymer implanted in rabbit corneas sharply curtailed maximum blood vessel length, compared to empty pellets.



"These results demonstrate that basking shark cartilage extract strongly inhibits tumor-induced neovascularization, and that significant inhibition can be obtained with the extracts at a crude stage of purification," states Professor Langer. In contrast, calf cartilage extract must be highly purified to slow down vascular growth. He feels that with further study, shark cartilage may become a major source of angiogenesis inhibitor.

Basking sharks can weigh 10 times more than calves and have over 10 times greater percentage of cartilage as their body weight. In addition, comparable antineovascular activity can be extracted with 1000 times less shark cartilage. Thus, on a per-animal basis, sharks possess 100,000 times more anti-vascular activity than calves. "This result has broad implications for the entire future of tumor neovascularization research and could make sharks an abundant source of a new class of therapeutic agents," says Professor Langer.

Identifying this factor might also provide insights into the tissue development of different species and may contribute to understanding why elasmobranchs such as sharks so rarely exhibit neoplasms compared to mammals, bony fish, reptiles, and amphibians. It could also provide a new use for sharks, currently an undesirable catch for fishermen.

The long term goal of the angiogenesis research is to completely identify the chemical entity responsible for inhibiting neovascularization. Two principal problems have limited the purification of this inhibitor. First, the supply of the active agent is very small, "although the shark cartilage studies go a long way toward solving this problem," adds Professor Langer. The second problem is the lack of a rapid, sensitive assay to follow the purification of the shark-derived inhibitor. The current assay involves using rabbit corneas, takes 30 days, and requires 12 rabbits to test one sample.

"It would be much better if we could get an answer in one day and test hundreds of samples," says Professor Langer. "We are trying to develop in vitro assays, such as some new methods based on capillary cells in culture, so that we will not have to use whole animals.

"We proposed that the improved assays for the inhibitor should be based on the in vitro simulation of a critical physiologial in vivo event in the process of neovascularization," says Professor Langer. Such an inhibitor could work in either of two ways: it may inhibit the proliferation of capillary endothelial cells; or it may inhibit the migration of capillary endothelial cells.

Students currently working on the project include Jennifer Behm, Kirsi Alison, and Mina Van Beuzokom; Anne Lee was involved previously.

3.0 BIOLOGICAL MONITORING OF AQUATIC ENVIRONMENTS BY DNA HYBRIDIZATION ASSAYS FOR COLIFORMS

Every summer, public health departments regularly check the number of coliform bacteria in the water at ocean and lake beaches. When the number of bacteria rises too high, indicating that the water is contaminated by sewage or sludge discharges, the beaches must be closed and swimmers are drydocked.

"The microbiological assay for detecting and tabulating coliform is rather insensitive, laborious, and time-consuming," says Professor Renee Fitts of the MIT Department of Applied Biology. In contrast, recent advances in DNA probe technology make it possible to detect potentially any organism in water, sewage, or other biological samples. The probes, which may consist of chromosomal genes or plasmid-borne genes encoding toxins or virulence factors, are used in DNA hybridization assays and can reveal the presence of very minute quantities of the sought-for organism.

Similarly, the detection of other microbiological human health hazards in the ocean, such as the red tide organism (shellfish poisoning), the hepatitis A virus, and V. parahaemolyticus (which causes severe diarrhea), is so laborious and expensive that it is often simply not done. DNA probes and hybridization assays, on the other hand, provide an excellent, economically feasible tool for specifically identifying and quantifying microbes in complex environments, says Dr. Fitts. Her work in this area has won her the 1986 Doherty Professorship at MIT, a two-year appointment to conduct research under the aegis of the MIT Sea Grant Program.

Traditional methods for assaying bacterial populations in water samples include 1) counting under a microscope the number of organisms present; 2) allowing the bacteria to grow into colonies; or 3) measuring total DNA or ATP in the samples. None of these methods, though, specifically identifies the organisms present. With a carefully chosen probe, which may consist of chromosomal genes or plasmid-borne genes encoding toxins or virulence factors, a DNA hybridization assay allows the researcher to discern and count any particular organism.

The development of DNA probes for coliforms would be the first application of this technology to ocean pollution problems. Dr. Fitts says, "We have developed diagnostic assays for the presence of bacterial pathogens in foods, resulting in a commercially available test for the food industry which has shortened their salmonella testing procedure by several days. This proposed Sea Grant project addresses similar problems in water resources, and might also lead to commercially available products for managing coastal waters."

To perform a DNA hybridization on a particular organism such as E. coli (the most prevalent coliform in the human colon and therefore in sewage), the researcher carefully chooses a segment of that organisms's DNA to use as a probe. The probe DNA must consist of a nucleotide sequence found in all isolates of E. coli but not in other organisms. Next, the researcher develops

a method for handling the sample. For example, testing a water sample from a harbor into which sewage has been released can involve collecting the bacteria in the sample onto a membrane filter.

The DNA in the bacteria which are stuck to the filter is released from the bacteria by adding a simple reagent. Then the DNA is denatured into single DNA strands and permanently stuck to the filter, so it will not wash away when the filters are soaked in a hybridization mixture containing probe DNA. The probe molecule seeks out complementary sequences in the target DNA on the filter and base-pairs with it to form a hybrid duplex. Since the probe molecule is always labeled in some way, usually with a radioisotope, the amount of E. coli in the water sample is calculated by simply measuring radioactivity on the hybridized filter.

"We also want to isolate a DNA probe that will detect coliforms other than E. coli. Although E. coli is the most prevalent coliform, it is not the only one. If we can detect other organisms, we will have a better index of sewage contamination and we will have a much more sensitive assay," says Dr. Fitts.

Isolating a DNA probe specific for E. coli, and additional probes for other organisms which are health hazards, would make it possible to locate and count these organisms throughout Massachusetts Bay and the New England coastline. These techniques would be extremely useful to public health laboratories, environmental protection workers, researchers interested in aquatic microbial populations, laboratories performing coliform counts on water samples, and some commercial fishing ventures. In addition, DNA probes and hybridization assays are especially amenable to automation and are attractive for further development as products for biotechnology companies.

A hybridization assay has two components: the DNA probe sequence itself and the method of labeling so that the probe molecule can be detected in the assay. This problem has received considerable attention in the biotechnology industry. "We can undoubtedly look forward to much more sensitive detection systems in the future," thinks Dr. Fitts. "We will show how DNA probes can be used to monitor bacteria in the ocean. The next step is to automate the assay, which will undoubtedly include development of new detection systems and instrumentation."

Dr. Fitts and colleagues plan to study several thousand water samples from the New England coastline using their E. coli specific probe and presumptive coliform-specific probe in hybridization assays. In parallel, they will perform standard microbiological assays for the presence of coliforms to determine the relative efficiencies of the different methods and evaluate any possible discrepancies.

"The only really time-consuming part of the study will be the microbiology, since the hybridizations can be done in batches very easily in a short time," says Dr. Fitts. "The advantages of hybridization assays will become clear at this point, because of the ease of handling samples and the tremendous savings in time and effort." Initially they will collect water samples at

shorelines. When the efficacy of the technique is clear, they can consider using this technique to study dispersion of the bacteria further in the ocean.

"The major benefit of a DNA hybridization for the presence of coliforms in coastal waters is a great time saving and cost saving in the performance of coliform counts, mainly by public health laboratories," remarks Dr. Fitts. "Since these assays are done to evaluate health and environmental hazards, the end result of a faster, better test is better management of water resources from both commercial and recreational standpoints."

4.0 USING MARINE MATERIALS FOR CONTROLLED RELEASE TECHNOLOGY: POLYMERS IN MEDICINE, AGRICULTURE AND FOOD

For many years, drug related research has focused on discovering potent compounds with new kinds of biological activity. While this research area continues to be important, increasing attention is being paid to how these drugs are delivered. Methods under consideration include incorporating drugs into solid polymers, or into controlled release devices using polymers.

Marine plants and animals constitute a unique, abundant source of biopolymers in a wide range of molecular weights, polarities and charge densities. Even though current applications of these polymers are limited, their versatility and biological compatibility provide a basis for many valuable uses. Particularly exciting areas are controlled release of drugs, controlling diffusion in food applications and other biotechnological applications, says Professor Marcus Karel of the MIT Department of Applied Biological Sciences. He will report on work done in collaboration with Professor Robert Langer of the same department.

Controlled release of pharmaceutical agents has many advantages over the conventional peak-and-valley release, exemplified by injecting daily doses of insulin. Maintaining the drug blood level in a precisely determined range over a prolonged period can eliminate the numerous side effects caused by the dosage peaking right after administration and ebbing below the level of effectiveness right before the next shot. Systemic effects are minimized by targeting the controlled release drug to a particular organ, such as releasing medicine for glaucoma directly in the eye. The rate-limiting factor for drug release is its removal from the polymer, rather than a patient deciding to take less because of side effects or taking more in hopes of hastening recovery.

Patients who might forget to swallow an assortment of pills several times daily can easily comply with an elaborate drug regimen with implanted controlled release drugs that work for weeks. Protected from the surrounding environment by a polymer, the drug's lifetime in the body is prolonged. Often less drug is needed, possibly with fewer side effects, since it is precisely targeted and its lifetime is improved.

In the past decade, controlled release systems have been introduced for eye diseases, motion sickness, birth control, asthma and heart disease. However, all the systems developed so far have been designed for small molecules. Large molecular weight drugs are not easy to deliver orally because they are degraded by acid or enzymes and have difficulty being absorbed, while their size prevents permeation through the skin. Delivered subcutaneously, their half lives are often less than 20 minutes. Systems are needed that can be injected under the skin and release large molecular weight drugs for long periods at controlled rates.

A controlled release system must be nontoxic, noncarcinogenic, nonteratogenic, and have no other undesirable effects. It should incorporate drugs made of large molecules without significantly denaturing them or otherwise altering their biological activity. So far very few systems have been designed with these properties in mind, and fewer still are biodegradable.

"We believe that materials from the sea are potentially promising for delivering macromolecules or pharmaceutical agents," says Professor Karel. In particular, alginate and carrageenan can be gelled in the presence of a desired pharmaceutical agent just by introducing an ion. Mammalian cells can be put in alginate and gelled simply by adding a calcium salt, and the cells will be maintained completely intact. A similar approach can be used with carrageenan. In contrast, many synthetic polymers require harsh treatments such as heat or solvents, which could possibly denature the substance to be incorporated.

Substances can also be easily formed into injectable microcapsules by similar methods. Carrageenan and alginates are widely available and often inexpensive, and initial data suggest that some will have very good toxicological properties. Both carrageenan and alginate have already been approved by the FDA for use in foods.

Controlled release polymers used in pesticides and fertilizers have greatly affected the agricultural industry. Several controlled-release pesticides are being used effectively against cockroaches and other insects, and studies have shown that these pesticides are less dangerous to other animals which inadvertantly become contaminated.

Another use of controlled-release technology is pheromones for insect control. These volatile sex attractants, which insects can sense in unbelievably tiny quantities over long distances, are put in polymer bait to attract and trap insects. Once caught, the insects can be killed effectively without having to spray whole areas.

A third major field for controlled release technology is the food industry. The most important applications of controlled release polymer technology in foods include the following:

1. Encapsulation and controlled release during preparation or consumption of natural and/or synthetic flavor compounds (by far the most extensive application of controlled release in the food industry).

2. Shielding oxygen- or water-sensitive compounds such as flavor, vitamins and other biologically active ingredients.

3. Isolating reactive or catalytic ingredients until their release is desired. Iron supplements in enriched foods are encapsulated to prevent them from making fats rancid, and to avoid discoloration from reactions with sulfur compounds. Leavening agents can be encapsulated to be released at appropriate times during baking.

In many food technology applications for preservatives, treatments are needed to enhance surface resistance to microbial growth. One approach is to maintain an unequal preservative distribution, or to start with a higher initial concentration of preservative(s) on the surface and use a coating to maintain this concentration difference for as long as possible. This approach requires a coating capable of reducing the diffusion of preservatives from food surface into food bulk.

Improved stability of surfaces can also be achieved by lowering surface pH. For lipophilic acids commonly used as preservatives, such as sorbic acid, surface pH reduction increases surface availability of the most effective form of the preservative (undissociated acid).

Marine biopolymers are uniquely suited to enhance surface resistance to bacterial growth and also to lower surface pH. Preliminary work with marine colloids agarose and carrageenan explored for the first time the possibility of stabilizing foods by maintaining a surface pH different from the desired bulk pH. Local pH control is particularly important in using food perservatives on food surfaces, because for the most widely used preservatives (propionates, benzoates, sorbates), the preservative effectiveness changes with pH in the range of 5 to 7 by a factor of 10 with each pH unit.

Professor Karel mentions that experiments to evaluate the effectiveness of marine biopolymers as perservative carriers and surface pH controlling agents will be conducted on a cheese analog, which represents highly sensitive foods requiring surface production. The coating will be chosen from agarose, alginates, and carrageenan with the aim of attaining the necessary surface pH and surface concentration of preservatives. These experiments will develop guidelines to formulate marine polymer based coatings to enhance the stability of foods, especially items to be distributed unfrozen and without prior sterilization.

5.0 RECENT DEVELOPMENTS IN CHITIN PRODUCTION AND APPLICATION

Shellfish, insect exoskeletons, and the cell walls of mushrooms and fungi contain appreciable amounts of chitin, a material rather like cellulose. (See Figure 3.) Billions of tons of chitin (pronounced kite-in) are synthesized annually by plants and animals. Encouraged by the growing number of applications for chitin and its derivatives in areas ranging from adhesives to water pollution control, a number of companies are now actively studying this abundant substance.

Increasing numbers of biological processes are being discovered in which chitin and related compounds play significant roles. Dr. E. Ray Pariser, Associate Director for Education in the MIT Sea Grant Program and author of the Chitin Sourcebook, says, "Insight into these roles continues to reveal new properties of the polymer and greatly contributes to the development of new and potentially useful industrial applications. Chitin and its derivative chitosan are materials of great scientific and practical interest today."

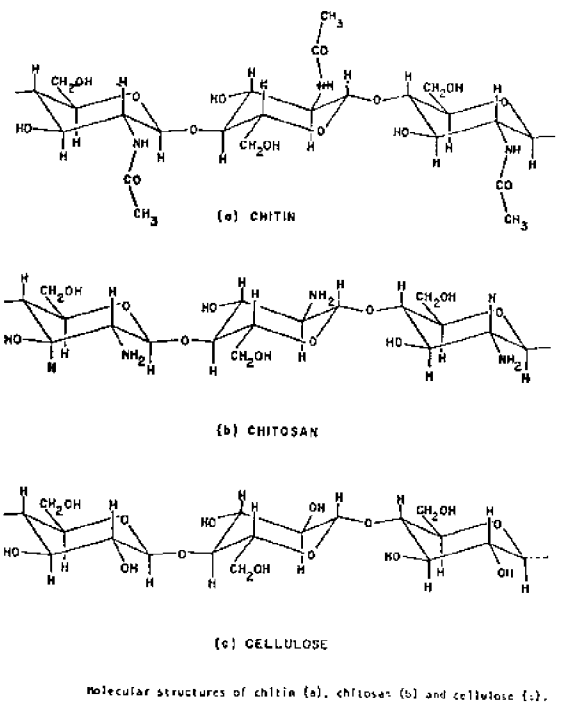
Chitin is a straight-chain polymer of N-acetyl-glucosamine. Differences in chain length, degree of acetylation, amino acid content, etc., have been observed in chitins and chitosans (de-acetylated chitin) obtained from different organisms. (See Figure 4.) These natural differences have made it difficult to produce chitin end products of consistent properties and performance.

Supported by the Sea Grant College Program, MIT researchers in several departments have studied chitin for many years. Professor Benjamin Averbach of the Department of Materials Science and Engineering investigated techniques for casting chitosan membranes and the metal chelating ability of chitosan. In the Department of Applied Biological Sciences, Dr. Cho Kyun Rha is studying the possibility of using chitosan globules to separate the products of biotechnological processes from the specialized bacteria or yeast cells which make them. Researchers from the Biomaterials Science and Engineering Laboratory fabricate chitosan membranes for possible use as artificial blood vessels. Important discoveries illuminating the nature, synthesis and use of chitin and derivatives have been made in other Sea Grant supported research centers, including the Universities of Delaware, Washington, and Georgia.

Adhesives were an early application for chitosan. During World War II, the British used a chitosan glue derived from insects to hold together the wooden parts of the Mosquito fighter/bomber aircraft. Chitosan-based cements have been successfully used to join and produce laminate with combinations of wood, paper, cotton and other materials. A small amount of chitin increases both wet and dry strength of paper.

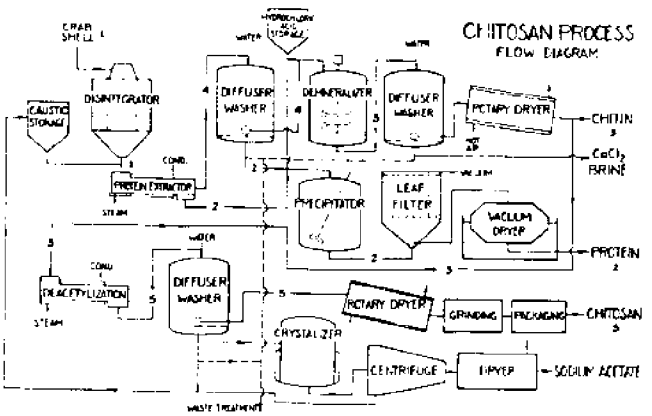
More recently, it has been discovered that chitin in purified form may be inserted under the skin or in contact with body fluids without harm. Tissue enzymes slowly attack the chitin until the body eventually absorbs it. Its ability to accelerate the natural fusion and healing of incisions and other

Figure 3. Molecular structure of chitin, chitosan, and cellulose.



Molecular structures of chitin (a), chitosan (b) and cellulose (c).

Figure 4. Chitin-chitosan flow chart.



Chitin-chitosan process flow chart. Number key: (1) shell, (2) protein, (3) chitin, (4) deproteinized shell, (5) chitosan.

wounds has led to studies of chitin for wound treatment, in temporary skin prostheses, in surgical sutures, and for other surgical aids. So far, these efforts have resulted in several U.S. patents and new drug applications from the FDA. The phenomenon of slow adsorption has also sparked interest in using chitin or degradable derivatives as materials for controlling the slow release of drugs within the body or in special external locations such as the eye.

The positive charge of the chitosan molecule lets it flocculate very dilute aqueous solutions of proteins. Small amounts (parts per million) of chitosan can thus purify the effluent from food processing plants by removing the protein, which is then recovered and fed back to animals. This is the first application for the use of chitin approved by the FDA.

Chitosan powder apparently helps protect seeds from mold and rot. Experiments at the University of Washington gave such encouraging results that a large test involving thousands of acres of winter wheat on the West Coast is currently in progress, sponsored by the U.S. Department of Agriculture.

Billions of pounds of whey are produced yearly as a byproduct of cheese manufacturing, and represent a serious waste disposal problem. Most animals (including humans) cannot digest lactose after infancy, so the whey with its 70% lactose cannot be freely added to food or livestock feed. Lactase, the enzyme needed to digest lactose in milk, is produced by a microorganism which thrives on a chitin base. Some chicken feeds now contain a little chitin to enhance the growth of this microorganism. The lactase thus produced makes it possible for the chickens to digest the whey.

Chitin and chitosan development and production mechanisms are also important because researchers want to get away from depending on shrimp waste shells as the sole raw material. Chitin is found in the outer cell wall of molds and fungi, suggesting that it could be cultivated under very controlled conditions. Dr. Pariser likens a hypothetical chitin farm to the manufacturing process for making cheese or beer.

Dr. Pariser and colleagues have recently formed the Chitin Company to take advantage of some of chitin's potential uses. The Company hopes to produce chitin and chitin derivatives of standard quality with consistent properties. "Varying raw material supplies represent a major obstacle in the industrial scale utilization of chitin. The development of new production methods using more easily controllable raw materials is therefore urgently required," he says.

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