Biomaterials and its Applications in Tissue Engineering

N. Gayathri\textsuperscript{1}, L. Nagarajan\textsuperscript{2}

\textsuperscript{1}M.Tech – II Year, Department of Textile Technology, Jaya Engineering College, Chennai – 602024
\textsuperscript{2}Associate Professor, Department of Textile Technology, Jaya Engineering College, Chennai – 602024

Abstract: Medical textiles are one of the rapidly growing sector in technical textile market. Medical Textile involves the combination of textile technology and medical sciences. Application of textiles in medicine includes extra corporeal, implantable, non-implantable, health and hygiene products. In implantable and extracorporeal devices, biomaterials are the base to produce the end products. Biomaterials can be Ceramics, Metals, and Polymers. In textile field, polymers are taken as a base material, from which woven/knitted/braided/nonwoven fabrics are produced for Medical applications. Tissue engineering is one of the emerging fields, in which biomaterial polymers are widely used. Mostly widely biodegradable polymers are used, and nanofibers are produced from that which is further converted into fabrics, to be used for tissue engineering applications. Nanofibers can be produced by both naturally occurring and synthetic polymers by various techniques like Rotary Jet Spinning method, Electrospinning, Centrifugal Spinning etc. When spun, the material stretches much like molten sugar does as it begins to dry into thin, silky ribbons. The most commonly used natural biopolymers for tissue engineering include silk, chitosan, collagen, etc. Synthetic polymers include degradable polymers such as, polymethylmethacrylate (PMMA), polylactic acid (PLA), polyurethane (PU) etc. The biodegradable nanofibers are used in the areas of tissue engineering such as neural, bone, cartilage, skeletal muscle, blood vessel, skin and ligament. Polymers possess great biocompatibility, good mechanical strength, good biodegradability, and it is used to replace diseased part, assists in healing, improves or corrects the function also assists in cell adhesion, growth, proliferation.

Keywords: Biomaterials, Biocompatibility, Tissue Engineering, Proliferation

1. Introduction

Biomaterials acts as 3D frames, which can be scaffolds, matrices or constructs. These 3D frames due to their high surface area to volume and their micro porous structure are used in cell binding, growth and differentiation. And as biodegradable nanofibers are used as scaffolds it eliminates the necessity of surgical removal. Hence the applications of nanofibers as scaffolds play an important role in tissue engineering. The potential of nanofibers is widely followed in biological and non-biological applications. This report deals with the fabrication and application of nanofibers in tissue engineering. Nanofibers have been used in all areas of tissue engineering such as bone, cartilage, ligament, skeletal muscle, vascular, neural and skin, and also it controls the delivery of drugs, proteins and DNA. Tissue engineering is an alternative method for organ transplantation, and its prime job is to (i) replace natural tissues characteristics, (ii) fill the space until damage tissue is regenerated, (iii) temporarily replace tissue function, (iv) to help in managing tissue growth. The main aim of tissue engineering is to repair or replace the damaged or diseased tissue.

2. Biomaterials

A natural or synthetic material (as a polymer or metal) that is suitable to introduce into living tissue especially as a part of medical device, to repair damaged or diseased parts.

2.1 Types of Biomaterials

1. Metals
2. Polymers
3. Ceramics

Polymers are classified into three types namely Natural and synthetic.

Natural Polymers
Natural polymers occur in nature and can be extracted. They are often water-based. Examples of naturally occurring polymers are Silk, Collagen, Chitosan, Alginate etc

Synthetic Polymers
Synthetic polymers are derived from petroleum oil, and made by scientists and engineers. Examples of synthetic polymers include PMMA, Polyethylene etc.

Biomaterials For Tissue Engineering
1) It has a good biodegradable property
2) It is used to replace diseased part
3) It assists in healing
4) It improves or corrects the function
5) It has a good biocompatibility and mechanical properties

2.2 Importance of Biomaterials

Toxicology
Biomaterial should not be toxic. Toxicology for biomaterials deals with the substance that migrates out of the biomaterials. It is reasonably to say that a biomaterial should not give off anything from its mass unless it is specifically designed to do so.

Biocompatibility
The ability of a material to perform with an appropriate host response in a specific application. Material should not bring out a prolonged inflammatory response.
Healing
Injury to tissue will stimulate an inflammatory reaction that leads to healing. When a foreign body is present in a wound site then it is known as foreign body reaction. Biomaterials assist for wound healing.

Functional Tissue Structure
Biomaterials are implanted into tissues and organs. The structure and role of biomaterial should mimic the natural one.

Biodegradability
The degraded products of the scaffold must have a safe route for removal from the host.

Mechanical And Performance Requirements
Biomaterials and devices must have mechanical and performance requirements that originate from the physical properties of the materials. The three categories of such requirements are mechanical performance, mechanical durability, and physical properties. The scaffold must be able to provide support to the forces applied to it and the surrounding tissues (especially true for the engineering of weight-bearing orthopaedic tissues).

3. Silk
Silk fibroin is a natural protein produced by the domestic silkworm. Biodegradability is the essential properties of biomaterial. Silk is popular for its luster and mechanic properties. It is a natural filament produced by silkworm. This contains fibrous protein termed as fibroin, and that forms sericin which helps to reinforce together. Removal of sericin from silk is done for biomedical, cosmetic, biotechnological, tissue engineering, drug delivery applications in order to prevent inflammatory. Silk has more mechanical strength and it has a very high strength and toughness.

Properties of Silk
- Supports in cell adhesion, growth, proliferation
- Excellent luster and good texture
- Excellent temperature retain ability
- High mechanical properties, high strength and excellent elasticity
- Very good tensile strength
- Good degradability and biocompatibility

Chitin and Chitosan
Chitin is a white, hard, inelastic, nitrogenous polysaccharide found in the outer skeleton of insects, crabs, and shrimps. It is basically obtained from prawn/crab shells. Chitosan is produced by chemically treating chitin. They both are suitable material for wound healing. They are natural polymers and they have excellent biodegradability, biocompatibility, non-toxicity and adsorption. They are also antibacterial and act against fungi. They possess low mechanical strength. They are more suitable for wound dressing materials as they have a very good wound healing property.

Collagen
Collagen is an important biomaterial in medical application for its excellent biocompatibility, biodegradability and weak antigenecity. Collagen makes up one quarter of all the total protein in the body. It possesses a fibrous structure and imparts strength to tissues such as tendons, ligament, and skin. It is very good to promote cell, tissue attachment and growth. It possesses low mechanical strength and has safety issues when derived from animals.

Poly (Methylmethacrylate)
It is a light weight synthetic material. It is alternate to polycarbonate, where extreme strength is not necessary. It does not contain potentially harmful substance. It possesses moderate properties, easy handling, processing and low cost. It has a good degree of compatibility with human tissue. In cosmetic surgery, PMMA injected under the skin to reduce wrinkles or scars permanently. It is resistant to inorganic solutions. It has excellent optical properties. It is suitable for Blood pump and reservoir, implantable ocular lenses, bone cement.

Polyvinylchloride (PVC)
It is amorphous & rigid polymer, have high melt viscosity. It is made flexible and soft by the addition of plasticizers. It is suitable for blood and solution bag, surgical packaging.
Polyurethanes (PU)
They have excellent mechanical properties and good biocompatibility. They are used in the fabrication of medical implants such as vascular grafts. Polyurethanes can be designed to have chemical linkages that are degradable in a biological environment. They are very much suitable for tissue engineering applications because of their mechanical properties and good biocompatibility.

4. Applications of Tissue Engineering

Tissue engineering is widely used in the fields as mentioned below.

4.1 Bone Tissue Engineering

It is based on physical properties of scaffolds such as mechanical strength, pore size, porosity, hardness and 3D architecture etc. Scaffolds with greater porosity are preferred for better cell/tissue-in-growth and enhanced bone regeneration. Studies have shown that PCL based nanofibrous scaffolds are potential candidates for skin tissue engineering.

4.2 Cartilage Tissue Engineering

PCL based nanofibrous scaffolds by electrospinning are the most suitable one for this. It possesses very good mechanical properties. It has a good degradation, cell attachment and growth factor delivery.

4.3 Ligament Tissue Engineering

They are connective tissue responsible for joint movement and stability. Ligament ruptures result in damage of connective tissues leading to tissue degenerative diseases, which do not heal normally and cannot repair by clinical methods. PU nanofiber aligned tissue engineering successfully meet this challenge.
4.4 Skin Tissue Engineering

Skin wounds heal naturally by formation by epithelialized scar tissue rather than by regeneration of full skin. Of the two of d layers of skin, epidermis and dermis, has less capacity to heal. But when large areas to be heal, normal regeneration is lacking, hence epidermis to be replaced. The scar tissue that forms in the absence of dermis lacks elasticity, flexibility and strength. Scar tissue causes pain, limit movements.

For this, skin tissue engineering is the very good alternative to stimulate the regeneration of dermis. Nonwoven based silk nanofibers by electrospinning method are much suitable for skin tissue engineering due to their high porosity and high surface area to volume. And also this coated with type I collagen is suitable to promote cell adhesion and spreading.

4.5 Blood Vessel Tissue Engineering

Conventional spinning method produces randomly oriented nanofibers. Biodegradable PLLA-CL nanofibers explored to fabricate tubular scaffolds that could be used for blood vessel. They mimic the natural ECM, provide mechanical properties and give a good architecture which helps for cell adhesion and proliferation.

5. Applications of Tissue Engineering With Suitable Polymers
| Method                          | Polymers                          | Unique factors                                  | Application                                                                 |
|--------------------------------|-----------------------------------|-------------------------------------------------|-----------------------------------------------------------------------------|
| Hydrogel scaffold fabrication  | Alginate, PMMA, HA, PEG           | Microgels, biologically degradable, mechanical and physical Complexity | Insulin delivery, gene therapy, bioreactor, and immunosolation              |
| Micromolding [61–63]           | Chitosan, fibronecin, HA, PEG, PMMA, PAA, PMMA, PAAm, and PDMAEM | Microspheres, microarrays, controlled size and shape | Micro-devices, biosensors, growth factors, matrix components, forces, and cell-cell interactions |
| Photolithography [64–66]       | PEG, PEG, calcium alginate, silicon and PDMS | Microbeads, microvials, valves, and pumps | Sensing, cell separation, cell-based microreactors, and controlled microreactors. |
| Microfluidics [67–69]          | Gelatin, HA, and collagen         | Microgels, microsensors, cell-based diagnostics | Sustainable and controllable drug delivery therapies                       |
| Acellular scaffold fabrication |                                   |                                                 |                                                                             |
| Decellularization process [73–75] | Biological tissues              | Retain anatomical structure, native ECM, and similar biomechanical properties | Tissue engineering                                                        |
| Keratin scaffold fabrication   |                                   |                                                 |                                                                             |
| Self-assembled process [76–78] | Keratin                          | Bio-compatibility                               | Drug delivery, wound healing, soft tissue augmentation, synthetic skin, coatings for implants, and scaffolds for tissue engineering |
| Fibrous scaffold fabrication   |                                   |                                                 |                                                                             |
| Nanofiber electro-spinning      | PGA, PLA, PLGA, PCL              | High surface area, biomechanical, and biocompatibility | Drug delivery, wound healing, soft tissue synthetic skin, and scaffolds for tissue engineering |
| process [79–81]                | copolymers, collagen, elastin, and so forth | | Solar sails, reinforcement, vascular grafts, nonwetting textile surfaces, and scaffolds for tissue |
| Microfiber wet-spinning         | PLGA, PLA, chitosan, and PCL      | Biocompatible fibres with good mechanical properties | Filtration, membrane separation, protective military clothing, biosensors, wound dressings, and scaffolds for tissue engineering |
| process [82–84]                |                                   |                                                 |                                                                             |
| Nonwoven fibre by melt-blown    | Polyessters, PGA, and PDO         | Submicron fiber size, highly porous scaffold    | Drug delivery, wound healing, soft tissue synthetic skin, and scaffolds for tissue engineering |
| process [85–87]                |                                   |                                                 |                                                                             |
| Functional scaffold fabrication |                                   |                                                 |                                                                             |
| Growth factor’s release process | Collagen, gelatin, alginate,     | Membranes, hydrogels, foams, microspheres, and particles | Angiogenesis, bone regeneration, and wound healing |
| [88–90]                        | chitosan, fibrin, PLGA, PLA, and PEG | |                                                                             |
| Ceramic scaffold fabrication    |                                   |                                                 |                                                                             |
| Sponge replication method [91–93] | PU sponge, FVA, TCP, BCP or     | Interconnected porous ceramic scaffolds          | Bone tissue engineering                                                   |
|                                 | calcium sulfite                   |                                                 |                                                                             |
|                                 | Coating on: metals, glasses, inorganic ceramics and organic polymers (PLGA, P, PEG, silicones and PTFE), collagen, fibres of silk, and hairs | |                                                                             |
| Simple calcium phosphate        |                                   | Improve bio-compatibility or enhance the bioactivity | Orthopedic application                                                   |
| coating method [94–96]          |                                   |                                                 |                                                                             |
| Automation and direct organ     |                                   |                                                 |                                                                             |
| fabrication                     | Sodium alginate                  | To build complex tissues composed of multiple cell types (Hydrogel scaffold) | Biosensor development, microdeposition of active proteins on cellulose, biochips and acellular polymeric scaffolds |
| Injet printing process [97–100] | Biodegradable polymers or blends | Complex 3D solid object, good mechanical strength | Honey comb structure scaffold, hard-tissue scaffolds |
| Multibased rapid prototyping    |                                   | Design and fabrication of patient-specific scaffolds and automated scaffold assembly algorithm | Develop a program algorithm that can be used to design scaffold internal architectures |
| process [101, 102]             |                                   |                                                 |                                                                             |
| Computer-aided design (CAD)     |                                   | Layer by layer deposition of cells or matrix | To print complex 3D organs with computer-controlled, |
| data manipulation techniques    |                                   |                                                 |                                                                             |
| [103–106]                      |                                   |                                                 |                                                                             |
| Organ printing [106, 107]       | Tubular collagen gel              |                                                 |                                                                             |
6. Research and Trends

Research in tissue engineering has revealed the design rules for joining cells with materials and for understanding the mechanism by which cellular functions can be influenced or interrogated by materials. Development of cell based micro-systems outside tissue engineering will in the long term provide technologies that will be applied to tissue engineering. The technology developed to integrate the functions of cells with electrical or mechanical processes in materials will have important benefits to the growth of tissue for transplantation and for prosthetic interfaces between indwelling devices and natural tissue.

Cell based engineering addresses the development of hybrid devices that combine cellular and tissue components with conventional materials and processes found in microfabrication. Research and development activities span a broad range of topics including technical development of methods and fabrication routes to join cells with materials, exploratory and discovery research to identify strategies for matching cellular processes with materials processes, and engineering of complete systems that exploit the unique realization that combining man made devices and biological systems.

Many consolidated findings that stem have enhanced the comprehension of molecular and process related to various diseases and physiological properties. Knowledge been applied to various types of medical treatments, such as creating intelligent biological drug system, and also helpful to better medical systems such as apoptosis and carcinogenesis. It must help us from mechanisms of aging, human development, and prevent from cancer, heart disease, mental illness, as well as several conditions. Hopefully it may help to successfully treat malignant disease in future.

Silk

**PROCEDURE FOR THE PRODUCTION OF SILK NANOFIBERS, TESTING AND REGENERATION OF GRAFT**

- **Degumming Silk**
- **Dissolving Silk**
- **Production of Silk nanofibers using Rotary Jet Spinning and testing the fibre**
- **Forming nonwoven matrix scaffold and testing**
- **Cell from biopsy**
- **Cell culture**
- **Expanded Cells**
- **Culture on a scaffold**
- **Generation of graft and testing**

**Degumming of Silk**
Silk is degummed with 1g/L of Na₂CO₃ for 45 minutes and 80°C and washed.

**Recipe**
MLR – 1:50
Na₂CO₃ - 1g/L
Soap – 5g/L

**Dissolving Silk In Libr**
50 ml of LiBr solution at 9.3M concentration, calculated amount of LiBr powder is 40.4 grams, which is dissolved in 32-33ml water. Then 2 grams of Silk is dissolved in 10 ml of 9.3M LiBr solution at 60°C for 4 hours in a closed container.

**Stirring Silk Using Magnetic Stirrer**
Silk solution is stirred using magnetic stirrer till it reaches the gel state.

**Silk Spinning Using Rotary Jet Spinning Method**
The prepared polymer solution gel is poured into the reservoir of the RJS machine. As soon as the machine is switched on, with the help of the controllable motor, reservoir starts to rotate. There is a hole made in the
reservoir. Due to the centrifugal force, polymer inside the reservoir ejects out and forms a thin ribbon like fibre structure, which is collected in the collector. The fibre forms its own path during rotation.

**PMMA**

PROCEDURE FOR THE PRODUCTION OF PMMA FIBRES, TESTING AND GENERATION OF GRAFT

[Image of a process diagram]

Stirring PMMA Using Magnetic Stirrer

2 grams of PMMA is taken with 10ml of Chloroform in a beaker. And it is stirred using magnetic stirrer till the polymers are completely dissolved in Chloroform and homogeneous mixture of solution is formed. The process is carried out till it reaches the gel state.

PMMA Spinning Using Rotary Jet Spinning Method

The prepared polymer solution gel is poured into the reservoir of the RJS machine. As soon as the machine is switched on, with the help of the controllable motor, reservoir starts to rotate. There is a hole made in the reservoir. Due to the centrifugal force, polymer inside the reservoir ejects out and forms a thin ribbon like fibre structure, which is collected in the collector. The fibre forms its own path during rotation.

7. Conclusion

Thus Silk has a very good biocompatibility, biodegradability, high surface area to volume, improved mechanical properties and hence very suitable for skin tissue engineering. PMMA are light weight, which does not contain potentially harmful substance, moderate properties, easy handling, easy processing and low cost, good degree of compatibility with human tissue. Hence it is very much suitable for craniofacial and bone tissue engineering. In cosmetic surgery, PMMA injected under the skin to reduce wrinkles or scars permanently.

References

[1] A.Ashwin Kumar, Karthick.K and K.P.Arumugam, (2011) ‘Properties of Biodegradable Polymers and Degradation for Sustainable Development’, Indian Journal of Chemical Engineering and Applications, Vol.2, No.3, June 2011
[2] BrahatheswaranDhandayuthapani, Yasuhiro Yoshida, Toru Mackawa and D.Sakthi Kumar,(2011) ‘Polymeric Scaffolds in Tissue Engineering Application’, International Journal of Polymer Science, Volume 2011, Article ID 290602, 19 pages, doi:10.1155/2011/290602
[3] Chalongprap TANGSADTHAKUN, SoradaKANOKPANONT, Neeracha SANCHAVANAKIT, Tanom BANAPRASERT, Siriporn DAMRONGSAKKUL, (2006), ‘Properties of Collagen/Chitosan Scaffolds for Skin Tissue Engineering’, Journal of Metals, Materials and Minerals, Vol.16, No.1, pp.37-44, 2006
[4] Clive Lee ‘Properties of Bone Cement: The Mechanical Properties of PMMA Bone Cement’
[5] EribertoBressan, Vittorio Favero, Chiara Gardin, Letizia Ferroni, Laura Iacobelli, Lorenzo Favero, Vincenzo Vindigni, Mario Berengo, Stefano Sivolella and Barbara Zavan, (2011), ‘Biopolymers for Hard and Soft Engineered Tissues: Application in Odontoiatric and Plastic Surgery Field, Polymers 2011, Vol.3, pp.509-526; doi:10.3390/polym3010509
[6] Fergal J.O’Brien, (2011), ‘Biomaterials and scaffolds for tissue engineering’, Materials today.2011; Vol.14, No.3
[7] Hetal Patel, MinalBonde, Ganga Srinivasan, (2011), ‘Biodegradable Polymer Scaffold for Tissue Engineering’, Trends Biomater.Artif. Organs, Vol.25, No.1, pp.20-29(2011)
[8] Jamie L.Ifkovits, B.S., and Jason A.Burdick, (2007), ‘Review: Photopolymerizable and Degradable Biomaterials for Tissue Engineering Applications’, Tissue Engineering, Volume 13, Number 10, 2007, DOI:10.1089/ten.2007.0093
[9] V.Kearns, A.C.Maclntosh, A.Crawford and P.V.Hatton, (2008), ‘Silk-based Biomaterials for Tissue Engineering’, Topics in Tissue Engineering, Vol.4, Eds.N.Ashammakhi, R Reis, & F Chiellini 2008
[10] Lie Ma, Changyou Gao, Zhengwei Mao, Jie Zhou, Jiacong Shen, Xueqing Hu, Chunmao Han, (2003), ‘Collagen/chitosan porous scaffolds with improved biostability for skin tissue engineering’, Biomaterials Vol.24, pp.4833-4841
[11] Macarena Peran, Maria Angel Garcia, Elena Lopez-Ruiz, Gema Jimenez and Juan Antonio Marchal, (2013), ‘How can Nanotechnology Help to Repair the Body? Advances in Cardiac, Skin, Bone, Cartilage and Nerve Tissue Regeneration’, Materials 2013, Vol.6, pp.1333-1359
[12] MerviPuska, Allan J.Aho and PekkaVallittu, ‘Polymer Composites for Bone Reconstruction’
[13] Milena Koleva, ‘Poly(methyl methacrylate) (PMMA)
[14] Ming Chen, Melissa Przyborowski, and Francois Berthiaume, (2009), ‘Stem Cells for Skin Tissue Engineering and Wound Healing’, Cris Rev Biomed Eng,2009, Vol.37, No.4-5, pp.399-421
[15] Muhammad Iqbal Sabir, Xiaoxue Xu, Li Li, (2009), J Master Sci(2009), Vol.44, pp.5713-5724
[16] Nandana Bhardwaj, Biman B Mandal, (2015), ‘Silk fibroin- keratin based 3D scaffold as a dermal substitute for skin tissue engineering’, Article in Integrative
Biology, Impact Factor: 3.76, DOI: 10.1039/C41B90045F

[17] Niels Grabbert, Bei Wang, Asaf Avnon, Shuyao Zhuo, Vitaliy Datsyuk, Svitlana Trotsenko, Piotr Mackowiak, Katrin Kaletta, Klaus-Dieter Lang, and Ha-Duong Ngo, (2014), ‘Mechanical Properties of Individual Composite Poly(methyl-methacrylate)-Multiwalled Carbon Nanotubes Nanofibers’, Materials Science and Engineering, Vol. 64 (2014) 012005, doi:10.1088/1757-899x/64/1/012005

[18] Pathiraja A. Gunatillake and Raju Adhikari, (2003), ‘Biodegradable Synthetic Polymers for Tissue Engineering’, European Cells and Materials, Vol. 5, pp. 1-16

[19] Quynh P. Pham, Upma Sharma, and Antonios G. Mikos, (2006), ‘Electrospinning of Polymeric Nanofibers for Tissue Engineering Applications: A Review’, Tissue Engineering, Volume 12, Number 5, 2006

[20] Rajesh Vasita, Dhirendra S. Katti, (2006), ‘Nanofibers and their applications in tissue engineering’, International Journal of Nanomedicine 2006;I(1), pp. 15-30

[21] Richard A. F. Clark, Kaustabh Ghosh and Marcia G. Tonnesen, (2007), ‘Tissue Engineering for Cutaneous Wounds’, Journal of Investigative Dermatology, Vol. 127

[22] Robert J. Kroezee, Marco N. Helder, Leon E. Govaert and Theo H. Smit, (2009), ‘Biomaterials Polymers in Bone Tissue Engineering’, Materials 2009, Vol. 2, pp. 833-856; doi:10.3390/ma2030833

[23] Rostislav V. Shevchenko, Stuart L. James and S. Elizabeth James, (2010), ‘A review of tissue-engineered skin bioconstructs available for skin reconstruction’, J. R. Soc. Interface (2010), Vol. 7, pp. 229-258

[24] Sheila MacNeil, (2008), ‘Biomaterials for tissue engineering of skin’, materialstoday May 2008, Vol. 11, No. 5

[25] I. O. Smith, X. H. Liu, L. A. Smith, and P. X. Ma, (2009), ‘Nano-structured polymer scaffolds for tissue engineering and regenerative medicine’, Wiley Interdiscip Rev Nanomed Nanobiotechnol. 2009 March; Vol. 1, No. 2, pp. 226-236, doi:10.1002/wnan.26.

[26] Weam F. Mousa, Masahiko Kobayashi, Masashi Neo, Takashi Nakamura, (2000), ‘Biological and Mechanical Properties of PMMA-based Bioactive Bone Cement’, Article in Biomaterials, Impact Factor: 8.56. DOI: 10.1016/S0142-9612(00)00097-1

[27] ‘Biodegradable Polymers for Tissue Engineering’, BEH. 462/3. 962 J Molecular Principles of Biomaterials

[28] ‘Tissue Engineering’, Nature by technology, Vol. 18, Supplement 2000