DESIGN AND DEVELOPMENT OF CHITOSAN-BASED THERMOGELS FOR TISSUE REPAIR AND THERAPEUTIC DELIVERY APPLICATIONS.

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Abstract

It has been demonstrated that the use of a moderate buffer such as ß-glycerophosphate (GP), allows the neutralization of chitosan solution up to physiological pH (pH ~ 7.2), without inducing any polymer aggregation or precipitation. Furthermore, the resulting solution can be kept in a liquid state at low temperatures, which spontaneously forms a homogeneous gel when heated up to body temperature or 37ºC. This chitosan-GP system, called BST-Gel™, is a novel family of injectable thermo-setting chitosan-based gels ideally suited for biomedical and pharmaceutical applications. Studies of various BST-Gel™ formulations have been shown to be highly biocompatible and appropriate for the encapsulation of living cells, the release of proteins and the design of in situ self-forming injectable implants. In addition to the known biological and wound repair benefits of chitosan, the ability to achieve thermogelling chitosan solution with physiological conditions opens the way to a new generation of minimally invasive therapeutic applications.

Introduction

Thermosensitive polymers have recently attracted much interest for designing aqueous formulations, which form drug delivery gels *in-situ*[1]. The most studied thermosensitive polymers are the poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) block copolymers, called poloxamers. They are the well-known thermoset gel-forming materials *in situ*[2-4]. Unfortunately, poloxamer gels are obtained at high concentration only (between 20 and 30%)[5] and they have been shown to erode rapidly[6], to be non-biodegradable and to induce unexpected increases in plasma cholesterol or triglycerides when injected intraperitoneally in rats[7,8]. However, block copolymers of ethylene oxide and lactic acid were proposed as alternative materials. They possess a better degradability and acceptance *in vivo*[9], but the main drawback associated with rapid erosion and high polymer concentration seems to remain.

A family of thermosensitive neutral solutions based on chitosan/glycerophosphate (chitosan-GP) combinations have been developed and proposed as an *in-situ* forming hydrogels[10,11], called BST-Gel™. Chitosan is a biocompatible and biodegradable copolymer of glucosamine and *N*-acetyl glucosamine[12-14], which is currently being investigated for many pharmaceutical applications[15,16]. Its digestibility by lysozymes has been shown to be dependent on the amount and the distribution of *N*-acetyl groups in the backbone chains[17-19]. Chitosans with a degree of deacetylation higher than 70% have been found to be soluble in acidic aqueous solutions. Once dissolved, chitosan remains in solution up to pH about 6.2, a value above which, chitosan precipitates. The chitosan-GP systems have unique properties which allow for chitosan solubility at physiological pH (6.8 to 7.2) in liquid form, and induce gelification when heated to body temperature for use as injectable scaffolds and vehicles for therapeutic delivery of cells and drugs.

We have shown that the subcutaneous or intramuscular injections of chitosan-GP formulations result in locally deposited solid implants. These implants degrade primarily as a function of the degree of deacetylation of the chitosan component, as well as implant size and location. The pH and temperature-dependent characteristics of chitosan-GP formulations make them superior candidates for the design of middle and long-term injectable delivery systems. The present report is meant to review the results collected from various studies performed on chitosan-GP formulations, and to reveal the potential of these formulations if used as delivery system for drugs, proteins or cells.

Biocompatibility and Biodegradation

Biocompatibility

Chitosan-GP based formulations were expected to have good biocompatibility, since the ingredients, in addition to water, are mainly chitosan and GP, both known to be safe and non-toxic. An extensive biocompatibility and toxicity GLP testing of chitosan-GP, including *in vitro* cytotoxicity, genotoxicity and hemocompatibility in conjunction with *in vivo* genotoxicity, intracutaneous reactivity and sensitization tests all yielded no negative findings (unpublished data).

Biodegradation

In-vivo (e.g. human body), chitosan is mainly degraded by lysozyme, an enzyme present in many tissues and secretions such as tears, saliva, blood and milk. Lysozyme is also released and utilized by phagocytic cells during the inflammatory response to a foreign implant. Our *In vitro* studies[20] showed a nearly linear relationship between chitosan DDA and degradation of by lysozyme (Fig. 1).

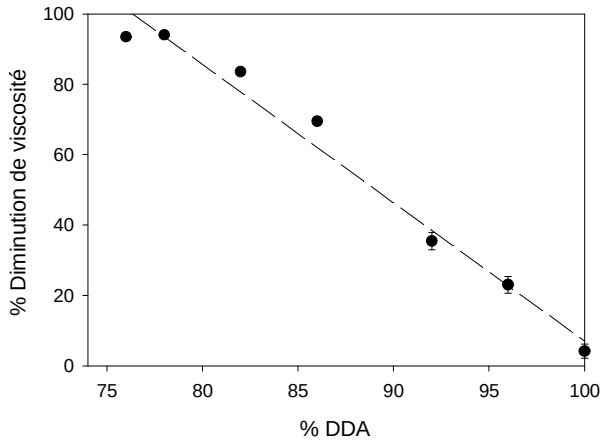

Figure 1 : *In vitro* degradation of chitosan by lysozymes monitored as loss of viscosity.

The fact that there is no covalent crosslink involved in chitosan-GP[10] suggested that degradation rates *in vivo* would be similar to that already known for chitosan. However, our studies showed that the biodegradation of chitosan-GP implants depends mainly on the chitosan DDA, the molecular weight, implant shape and location, and the local pH. Biodegradation of various formulations of chitosan-GP hydrogels was evaluated using 250 µL subcutaneous injections in rats. Histopathology and chitosan-specific staining[21] was used to analyze the state of the implant over time. Table 1 summarizes the *in vivo* subcutaneous characteristics of chitosan-GP implants over a period exceeding a year. This data along with the *in vitro* degradation profile allows customization of chitosan-based products in terms of desired residency times.

Table 1: *in vivo* subcutaneous biodegradation of chitosan-GP.

Chitosan DDA	95	92	86	79	75
Residency	> 1 year	> 1 year	~ 1 year	< 60 days	< 60 days

Properties of Chitosan-GP as delivery system

Drugs with various characteristics and therapeutic activities have been encapsulated in chitosan-GP gels. Three types of compounds, classified by their water-solubility, have been considered: soluble, poorly soluble and insoluble. For water-soluble compounds, the drug can be incorporated in chitosan-GP by simple dissolution, which leads to a homogeneous chitosan-GP/drug system. In contrast, poorly soluble or insoluble compounds which are incorporated as distinct phases which leads to a homogeneous biphasic formulation chitosan-GP /drug. Therefore, homogeneity is a key parameter, which must be assessed during the formulation of chitosan-GP gels for the delivery of such drugs. Other critical parameters are the drug stability and liberation profile as well as the injectability of the system.

Sol-Gel Transition

The gelation behavior of chitosan-GP/drug system was found to be quite similar to that of unloaded chitosan-GP without drug. Representative rheological data represented in Figure 2 shows the time dependence of elastic and viscous moduli. The elastic modulus (G') gives information about the gel stiffness and indicates the formation of a consistent gel within 10 minutes at 37°C.

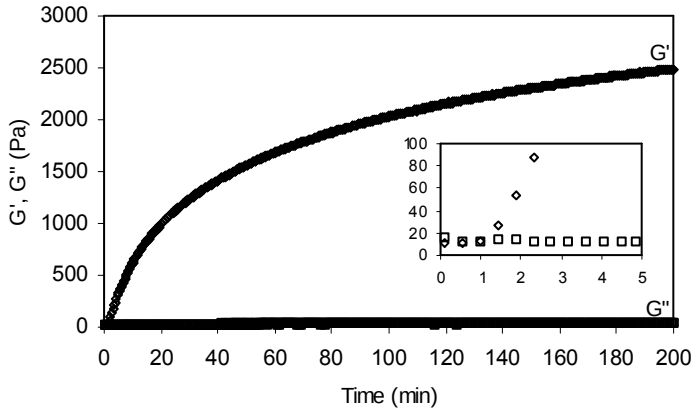

Figure 2 : Time dependence of elastic (G') and viscous (G") modulus at 37ºC, for a chitosan-GP formulation loaded with Hydrocortisone at concentration 5 mg/mL.

Homogeneity

The homogeneity of chitosan-GP/drug systems were determined with HPLC by analyzing samples taken from the upper, middle and lower portions of the preparation. Table 2 shows the results obtained for standard preparation containing 25% ($w_{drug}/w_{chitosan}$).

Table 2: Concentration of Hydrocortisone and Paclitaxel in drug loaded chitosan-GP preparations.

BST-Gel/Drug	top	middle	bottom	calculated
Hcort (mg/g)	1.79	1.78	1.78	1.81
Paclitaxel (mg/g)	1.79	1.79	1.80	1.81

Drug Stability

Drug stability was investigated using samples were withdrawn from the chitosan-GP/drug system periodically over time and analyzed using HPLC. Figure 3 demonstrates tat paclitaxel did not underg any changes over a period of 30 days in vitro.

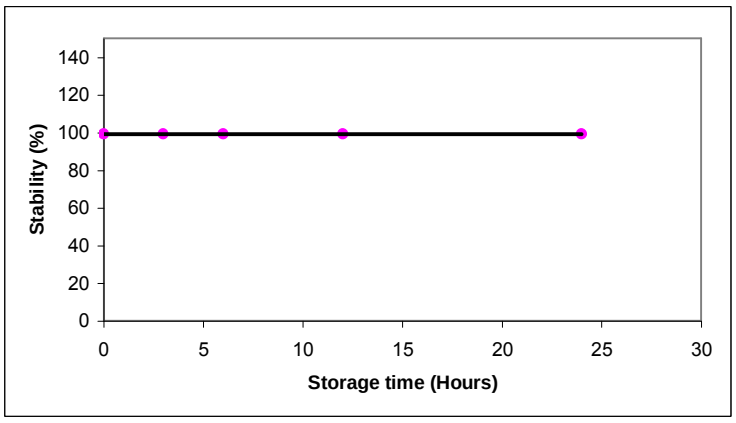

Figure 3 : Stability of paclitaxel after gelation of chitosan-GP/paclitaxel during storage at 25°C and 60% relative humidity.

Injectability

Injectability tests were performed where different formulations were injected through a 1 mL syringe equipped with a $26G^{5}/_{8}$ needle and strength needed to displace the syringe piston was measured using a micromechanical testing system (Mach-1^{TM}, Biosyntech). Drug loaded chitosan-GP formulations prior to gelation, chitosan-GP, chitosan-only solutions and chitosan/drug dispersions were tested. Figure 4 indicates that the injectability of chitosan-GP/drug formulations needs an average force of 600 mg, about six times greater than that needed for water, and estimated to be less than 10% of the force that can be exerted by normal individuals.

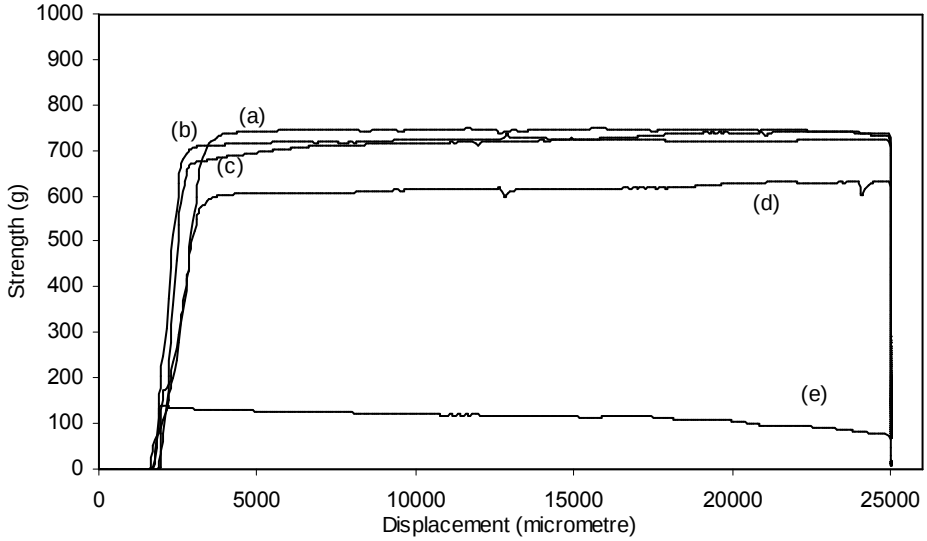

Figure 4 : Figure 4. Plot of the strength against the displacement of the piston for different preparations at a velocity of 800µm/sec for (a) chitosan-GP, (b) chitosan/drug dispersion, (c) chitosan solution, (d) chitosan-GP/drug and (e) water. Drug = hydrocortisone or paclitaxel.

Liberation profile

In vitro release profiles in PBS media recorded for various drugs encapsulated in chitosan-GP matrices show a dependency with the drug's solubility characteristic in water. Table 3 summarizes the results obtained.

Table 3: Release rates of drugs in PBS media

Compound	Therapeutic activity	Solubility in water (mg/mL)	Time to release 80%
Fluorouracil	**Antitumor**		**6 hours**
Pilocarpine hydrochloride	**Antiglaucoma**	**100**	**10 hours**
Tetracycline	**Antibacterial**	**1.7**	**3 – 4 days**
Hydrocortisone 21-acetate	**Glucocoticoid**	**0.01**	**1 – 2 weeks**
Camptothecin	**anticancer**	**Poorly soluble**	**4 – 5 weeks***
Paclitaxel	**Antitumor**	**Poorly soluble**	**5 – 6 weeks***

* The dissolution medium was supplemented by a surfactant SDS or Tween 20 to increase solubility in water. In the absence of the surfactant no drug was released even after 4 weeks.

In vivo applications

Chitosan-GP loaded with paclitaxel was injected intratumorally into EMT-6 tumors implanted subcutaneously on Balb/c mice. The local and sustained delivery of paclitaxel from the formulation revealed that the chitosan-GP/paclitaxel treatment has a significant ability to delay tumor growth, in a similar manner to Taxol® treatment alone (Figure 5). Furthermore, it showed that 1 intratumoral injection of the chitosan-GP containing paclitaxel was as efficacious as 4 intravenous injections of Taxol® in inhibiting the growth of EMT-6 cancer cells in mice, but in a less toxic manner. More details regarding this study can be found in Gariépy *et al*.[22].

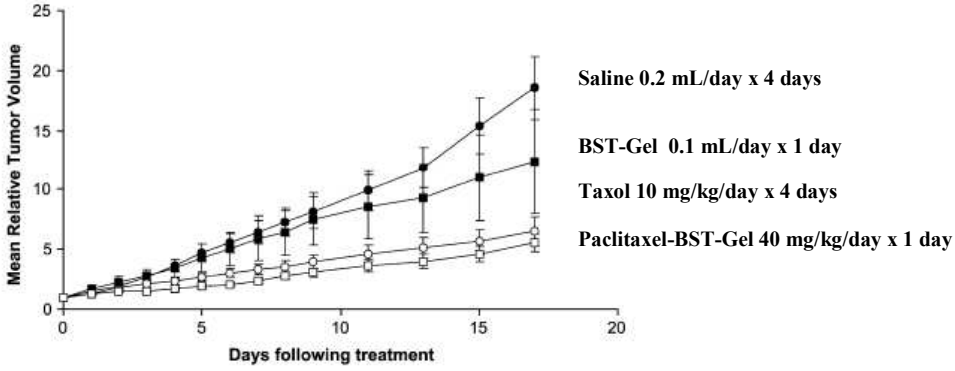

Figure 5 : In vivo anti-tumor effect for treatments initiated on day 4 after tumor inoculation (used with permission).

Recently, chitosan-GP loaded with an osteogenic protein proved to be effective in promoting bone formation. Indeed, two weeks after local injection of chitosan-GP containing BMP-2 under the tibial periosteum in rat resulted in a rapid bone proliferation. The newly formed bone was detected by radiology and evidenced by micro-CT images, as can be seen in Figure 6.

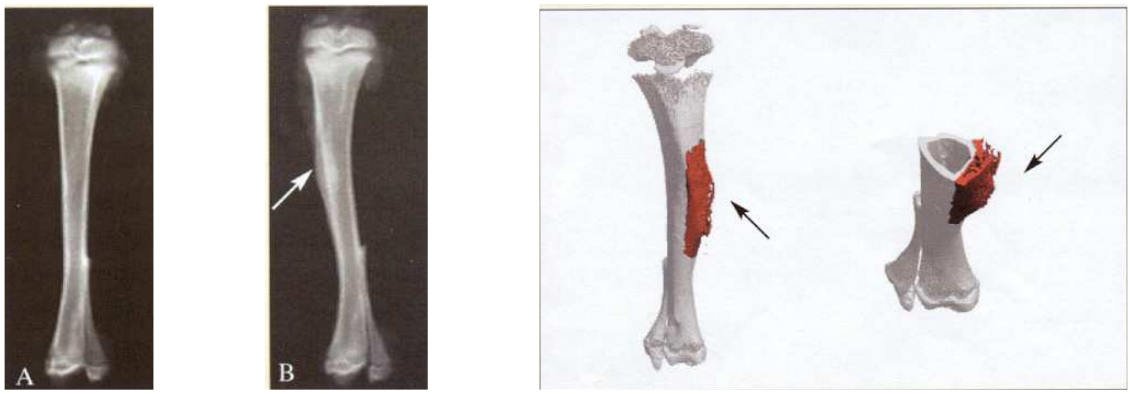

Figure 6 : *Ex vivo* radiographs (left) of the tibia periosteal reaction model in rats (A) Control (B) Treated animal. Three-dimensional micro-CT images (right) of a periosteal bone reaction (arrows): Whole tibia and Tibia image cut at the level of the maximal reaction.

Our studies have successfully showed the effectiveness of chitosan-GPhydrogels in tissue engineering and regenerative medicine applications, particularly in cartilage repair[23-25] and the treatment of inter-vertebral disc degeneration[26]. Other studies conducted by independent investigators have also evoked the relevance of the use of chitosan-GP hydrogel as a scaffold for nucleus pulposus cells[27] or stem cells[28] to treat inter-vertebral disc dysfunction.

Conclusion

This data taken together, shows that chitosan-GP has demonstrated superior performance as delivery device since it meets the known requirements needed for a successful therapeutic system. Among other things, the high level of biocompatibility, the large loading capacity and the biological applicability for drugs, cells and sensitive proteins puts chitosan-GP in a good position to satisfy the many biomedical needs.

References

[1] G. Chen, AS. Hoffman, Nature, 373, (1995) 49-52.

[2] TP. Johnston, SC. Miller, J. Parent. Sci. Technol., 43, (1989) 279-286.

[3] TP. Johnston, MA. Punjabi, CL. Froelich, Pharm. Res., 9, (1992) 425-434.

[4] EA. Pec, ZG. Wout, TP. Johnston, J. Pharm. Sci., 81, (1992) 626-630.

[5] SD. Desai, J. Blanchard, J. Pharm. Sci., 87, (1998) 226-230.

[6] R. Bhardwaj, J. Blanchard, J. Pharm. Sci., 85, (1996) 915-919.

[7] G. Chandrashekar, N. Udupa, J. Pharm. Pharmacol., 48, (1996) 669-674.

[8] WK. Palmer, EE. Emeson, TP. Johnston, Atherosclerosis, 136, (1998) 115-123.

[9] B. Jeong, YH. Bae, BS. Lee, SW. Kim, Nature, 388, (1997) 860-862.

[10] A. Chenite, C. Chaput, D. Wang, C. Combes, MD. Buschmann, CD. Hoemann, JC. Leroux, BL. Atkinson, F. Binette, A. Selamni, Biomaterials, 21(2000) 2155-2161.

[11] A. Chenite, MD. Buschmann, D. Wang, C. Chaput, N. Kandani, Carbohydr Polym, 46(2001) 39-47.

[12] KY. Lee, WS. Ha, WH. Park, Biomaterials, 16, (1995) 1211-1216.

[13] A. Miwa, A. Ishibe, M. Nakano, T. Yamahira, S. Itai, S. Jinno, H. Kawahara, Pharm Res, 15, (1998)1844-1850.

[14] O. Felt, P. Furrer, JM. Mayer, B. Plazonnet, P. Buri, R. Gurny, Int J. Pharm, 180, (1999) 185-193.

[15] L. Illum, SS. Davis, M. Pawula, AN. Fisher, DA. Barrett, NF. Farraj, PN. Shaw, Biopharm Drug Disp, 17, (1996) 717-724.

[16] VR. Patel, MM. Amiji, Pharm Res, 13, (1996) 588-593.

[17] S. Hirano, H. Tsuchida, N. Nagao, Biomaterials, 10, (1989) 574-576.

[18] S. Aiba, Int J. Biol Macromol, 14, (1992) 225-228.

[19] KM. Vårum, MM. Myrh, RJ. Hjerde, O. Smidsrød, Carbohydr Res, 299(1996) 99-101.

[20] S. Gori, Thesis (Masters of Applied Sciences in *Biomedical Engineering*), Ecole Polytechnique de Montréal, (2002).

[21] E. Rossomacha, C. Hoemann, M. Shive, Journal of Histotechnology 27(2004) 31-36.

[22] E. Ruel-Gariépy, M. Shive, A. Bichara, M. Berrada, D. Le Garrec, A. Chenite, JC. Leroux, Eur J. Pharm Biopharm, 57(2004) 53-63.

[23] CD. Hoemann, J. Sun, A. Légaré, MD. McKee, MD. Buschmann, Osteoarthritis and Cartilage, 13(2005) 318-329.

[24] CD. Hoemann, M. Hurtig, E. Rossomacha, J. Sun, A. Chevrier, M. Shive, MD. Buschmann, J. Bone Joint Surg Am., 87(2005) 2671-2686.

[25] Hoemann C, Sun J, McKee M*, et al.*, Osteoarthritis and Cartilage, (2006) in press.

[26] P. Roughley, C. Hoemann, E. DesRosiers, F. Mwale, J. Antoniou, M. Alini, Biomaterials, 27(2006) 388–396.

[27] WY. Su, L. Feng-Huei, 2nd World Congress on Regenerative Medicine, May 18-20, 2005, Leipzig, Germany.

[28] NL. Hughes, S. Richardson, AJ. Freemont, J. Hunt, JA. Hoyland, Eur Cell Mater, 10-Suppl-2 (2005) 12.