Effect of Molecular Weight of Chitosan Degraded by Microwave Irradiation on Bone Tissue Engineering Applications

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Statement of Purpose: Chitosan (CTS) is biocompatible, osteoconductive, degradable, and formable into 3D porous structures for bone tissue engineering and other applications [1]. The degree of deacetylation (DDA) and molecular weight (M_w) are two important properties of CTS [1]. Past studies have shown how different DDAs of CTS affect its physiochemical properties [2] and cell growth. While effects of M_w on CTS properties have been reported [3], their effects are not well known. It has been shown that microwave (MW) irradiation decreases M_w of CTS without affecting its DDA [4] providing the means to evaluate how M_w influences CTS properties while controlling DDA. The aim of this study was to evaluate the effect of M_w of CTS treated for 6, 18, and 30 mins by microwave irradiation on mechanical strength, crystallinity, and cell growth.

Materials and methods: 1% w/v solution of CTS (Vanson HaloSource, WA, 87.4% DDA) was prepared in 1% v/v HAc.

Microwave Treatment and Sample Preparation: CTS solution (500 mL beaker) was placed at the center of a commercial grade microwave (GE JES738WH 01), surrounded by 4x250mL conical flasks filled with water to maintain temperature below 100^{o}C. Samples were irradiated for 6, 18 or 30 mins at high power setting (700 W) in 2 min pulses. The water was replaced every 6 mins. MW treated CTS solutions were cooled to room temperature, frozen to -80^{0}C and lyophilized (LABCONCO FreeZone 2.5) for 48 hrs. 2M sodium hydroxide solution was added to neutralize the lyophilized CTS, followed by 3 washes with DI water. Samples were re-frozen at -80^{0}C and lyophilized for another 48 hrs.

NMR analysis: DDA of the MW treated CTS samples was verified using proton NMR (^{1}H NMR). Spectra were collected on a JEOL 270 MHz spectrometer at 70^{0}C [5]. 6s delay was used before application of pulse, acquisition time was 2s.

Molecular weight analysis: M_w of MW treated and untreated CTS solutions were determined viscometrically using the Mark-Houwink equation with $K=1.4x10^{-4}$dL/g and a=0.83 [6]. The viscosities of the CTS solution were measured in Cannon-Ubbelohde viscometers (9721-K50, CANNON Instrument Company) at 25^{o}C±0.1.

Mechanical Testing: Films were punched into ASTM E8 tensile testing specimens. Using an Instron 33R, model 4465 (Norwood, MA) Universal Testing Machine automated by Instron's Bluehill 2 (v2.13) software, the ultimate tensile strength (UTS) and Young's modulus of MW treated CTS films were determined. Constant tension rate of 1mm/min was used.

Crystallinity: The crystallinity index (CrI) of MW treated and untreated CTS powders were determined by X-Ray Diffractometry (Bruker D8 Advance, Cu-K_α radiation) and calculated using peak intensities at 2θ=20° and 2θ=16° [7].

In vitro studies: Growth of Saos-2 on CTS films was evaluated at 1, 3, 5 and 7 days via the CellTiter-Glo® Luminescent Cell Viability Assay. For the assay, cells were seeded at ~$5.0x10^4$ cells/wells in opaque 96 well plates (n=8 per CTS treatment group per day). Growth media (McCoy's 5A Medium + 10% FBS + 1% AB/AM) was used and renewed at 2 day interval. Results were normalized to controls.

Data were analyzed by ANOVA followed by SNK post-hoc tests. Significance declared at $p<0.01$.

Results and Discussion: ^{1}H NMR data confirmed that DDA of the CTS's was not affected by the MW treatment. ANOVA indicated significant reduction in M_w of CTS after 6 and 18 min of MW irradiation ($p<0.01$); no significant difference between 18 and 30 min ($0.01<p<0.05$). Negative exponential trend ($R^2=0.91$) was observed which plateaued quickly, at 18 to 30 min.

Table 1: Summary of physiochemical properties of MW treated CTS

MW Treatment	M_w (in10^5 g/mol)	Percent reduction	DDA %	CrI %	Modulus (GPa)
CTS	$4.40 \pm 0.11^{A*}$	-	87.74 ± 0.08^a	81.06	1.245 ± 0.119^b
6 min	4.11 ± 0.01^B	6.63	88.12 ± 0.26^a	ND#	1.546 ± 0.271^b
18 min	3.78 ± 0.01^C	13.96	87.58 ± 0.24^a	ND#	1.736 ± 0.392^b
30 min	3.77 ± 0.01^C	14.21	88.18 ± 0.05^a	ND#	1.495 ± 0.413^b

* groups with different letters are statistically different ($p<0.01$)
\# crystallinity index value not determined

ANOVA showed that there was no difference in elastic modulus of MW treated and non-treated CTS films ($p=0.025$) nor the ultimate tensile strength (UTS) (data not shown). XRD results indicated a loss of crystallinity of CTS samples with MW irradiation. 6 min MW treated CTS showed a large decrease in intensity of the 2θ peak at 20^0 (characteristic of CTS) and an increase in 2θ peak at 10^0 which prevented the calculation of the CrI (Table 1). There were no peaks identified in spectra for the 18 and 30 min MW treated groups indicating amorphous character.

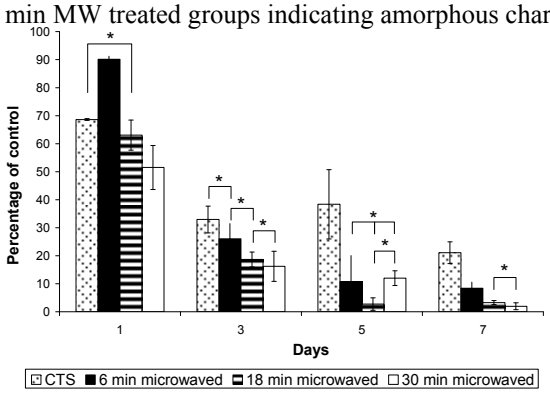

Fig 1: Growth study of Saos-2 using CellTiter-Glo® The results are normalized to the cells grown on TCP. The cells on control are proliferating faster than the CTS treatment groups; decrease in percentage values is seen for the treatment groups. *denotes no significance

ANOVA analysis revealed that there was a difference in the growth of cells between the CTS treatments ($p<0.01$) as well as a difference between days ($p<0.01$) (Fig 1). It has been reported that CTS with low M_w degrade faster than those with higher M_w [6,7]. It is speculated that the higher degradation rates lower MW CTS cannot provide a stable matrix for cells to grow and show poor cell growth characteristics.

Conclusions: MW treatment decreased M_w of CTS in an exponential manner without changing DDA. Increased times in MW irradiation resulted in decreased polymer crystallinity. Change in M_w resulted in no difference in elastic modulus, but a difference in cell growth of MW treated and untreated CTS films. Future studies will include degradation studies and effects on bone cell extracellular matrix production.

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Acknowledgements:

This work was supported by the Biomaterials Applications of Memphis (BAM) laboratories.