Synthesis of Raloxifene-Chitosan conjugate: A novel chitosan derivative as a potential targeting vehicle

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Abstract

Chitosan is a biocompatible, non toxic and biodegradable biopolymer. Due to the presence of functional groups on its surface, it can be modified and used as a carrier in targeted drug/gene delivery systems.

In this study, raloxifene (a selective estrogen receptor ligand) was conjugated to chitosan using different methods. The conjugates were investigated by means of FTIR, TGA and physical properties assessments. Cell viability was evaluated by XTT assay. FTIR and TGA results confirmed that the conjugation between chitosan and raloxifene occurred more efficiently when tri methyl chitosan in the presence of triethylamine and excess amount of linker was used. XTT assay on MCF-7 cell line illustrated that more than 80% of cells were viable after 24 hours exposure to selected molecules. These findings confirm that the conjugation of raloxifene-chitosan can occur successfully using special synthesis condition and this novel chitosan derivative can be introduced as a potential drug/gene targeting agent.

Key Words: *Targeting vehicle, Chitosan conjugate, Estrogen receptor, Raloxifene.*

Introduction:

Chitosan [α (1–4) 2-amino 2-deoxy β -D glucan], which is produced by deacetylation of chitin (Chitin is found in crustaceans shells) [1-3] is a linear polysaccharide comprised of glucosamine and N-acetylglucosamine units [4, 5]. This biopolymer and its derivatives possess distinct properties such as, biocompatibility, non toxicity, biodegradability [3, 6, 7] bioadhesivity [2] and antibacterial properties [2, 5].

Amino groups of Chitosan, which cause cationic property on its surface [1, 8-10], make it so attractive to use in biomedical applications such as drug/gene targeting purposes [4, 5, 7]. Moreover, presence of two functional groups (OH and NH₂) on Chitosan backbone make it chemically modifiable polymer [3, 5, 11, 12] in order to enhance the specificity of delivery or targeting system. A novel and potent method to enhance targeting of anti cancer drugs and therefore reduce their side effects along with improvement of therapeutic outcome is development of targeted drug delivery systems which can release the drug exactly at the desired site [13-15]. Three basic parts of these systems are: drug or bioactive agent, targeting moiety and a carrier [13].

Since tumor cells can over express some receptors or develop new specific ones, particular targeting moieties have been conjugated to the chitosan for achieving an acceptable tumor specific drug carrier and improve targeting potential of chitosan [16]. Folic acid [4], galactose [3] and FAP-B [17] are examples of these specific ligands.

Estrogens are groups of hormones which regulate growth and function of some tissues such as breast, uterus, ovarian and vagina [18]. Estrogen performs its function by binding to estrogen receptor (ER) [19]. Nearly 70%-75% of breast cancer cells express the estrogen receptor [20-22] that demonstrate the important role of estrogen in breast cancer cells growth [20, 22, 23]. Therefore by blocking ER function, risk of advance in breast cancer can be reduced.

Selective estrogen receptor modulators (SERMs), refers to a class of compounds that bind competitively with estrogen receptors and depending on the structure and position, act as agonist or antagonist of these receptors [21, 22, 24]. Raloxifene that has been received approval for prevention of breast cancer [24] is a drug which belongs to SERMs with antagonist activity in breast and uterus [23].

In this study a novel chitosan derivative was synthesized using different synthesis condition to utilize as an estrogen specific targeting vehicle. Raloxifene (as ER specific ligand) was

conjugated to chitosan via suitable linker and resulted derivative was characterized by means of FTIR and TGA. Moreover, its physical properties and cell toxicity were evaluated. The potential of this new chitosan derivative as a targeting nanoparticulate agent for anti cancer drug/gene delivery to breast cancer is performing in another study in our laboratory.

2. Materials and methods

2.1. Materials

Chitosan (Medium Molecular Weight (MMW-127 KDa) and Low Molecular Weight (LMW-70 KDa) was obtained from Primex (Island). Trimethyl chitosan (TMC, 30% quaternization), Raloxifene HCL, 3-Glycidyloxypropyltrimethoxysilane (3-GPTMS), XTT powder, PMS, dimethyl sulfoxide (DMSO), EDTA and Penicillin-streptomycin antibiotics were purchased from Sigma Aldrich. Triethylamine (Et_3N) and Chemical solvents were obtained from Merck (Germany). Dulbecco's modified eagle medium (DMEM), FBS (Fetal Bovine Serum), were obtained from Gibco (Germany).

2.2. Methods

In order to have a successful synthesis, choosing the appropriate condition is very important. To achieve the optimum synthesis parameters, 6 experiments were carried out by changing and evaluating of three important factors include chitosan type, linker/raloxifene ratio (mole to mole) and Et_3N amount (μl), (Table1).

2.2.1. Conjugation of drug to the linker

In order to conjugation of raloxifene to 3-GPTMS, raloxifene was dissolved in DMF and then dried toluene was added under the argon atmosphere. After 15 minutes, 3-GPTMS as a linker was added to mixture. Reaction was continued under reflux condition for 24h. All steps were done under magnetic stirring. The product of this step (linker-raloxifene conjugate) was a yellow color precipitate which was separated from the reaction mixture and washed thoroughly with methanol for eliminating the excess amount of epoxy linker.

2.2.2. Synthesis of drug-chitosan derivative

Firstly, a chitosan hydrogel solution (1%w/v) was prepared in acetic acid 1% (v/v). 7 ml of this hydrogel was added to drug-linker solution and stirred for 24h at room temperature. Then, organic and aqueous phases were separated and the study was continued using aqueous phase. Resulting mixture was dialyzed against sodium acetate buffer (pH: 4.8-5) for 72h in order to remove possible impurities and unreacted linker or raloxifene. At last, the product was dried at 110°C and subjected to further evaluations.

2.3. Characterization of synthesized molecules

2.3.1. FTIR studies

In order to evaluate the conjugation of linker to drug or drug to chitosan, IR spectra were recorded using KBr disks on a Magna-IR 550 Nicolet FTIR spectrometer.

2.3.2. Thermogravimetric analysis (TGA)

Thermogravimetric analysis curves of chitosan, raloxifene and resulted molecules were obtained in Nitrogen atmosphere, using a TGA (TGA 50 , SHIMADZU), with a heating rate of 10 °C/min, from room temperature to 595 °C.

2.3.3. Physical properties assessments

2.3.3.1. Glass transition (T_g) and Melting temperature (T_m) Determination

The melting / glass transition temperatures of raloxifene, chitosan and synthesized compounds were determined using open capillary method.

2.3.3.2. Solubility

The solubility of chitosans (LMW and TMC), raloxifene and synthesized products were studied using different organic and non organic solvents (Table2). The concentration of polymers was 1mg/ml in all experiments and the mixtures were heated to 60°C and sonicated for 30 seconds using an eurosonic 50/60Hz, 350W ultrasonication bath.

2.4. Cell Culture

MCF-7, a human breast cancer cell line used in this study were obtained from National Cell Bank of Iran (the Pasteur Institute of Iran, Tehran) and were cultured in DMEM supplemented with 10% (v/v) FBS, 100 µg/ml penicillin and 100 µg/ml streptomycin at 37°C in an incubator with 5% CO₂.

2.5. Cytotoxicity assay

Reduction of XTT (sodium 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)-carbonyl]-2H-tetrazolium inner salt), to its formazan derivative, is a common method to assay cell viability, cell proliferation and/or cytotoxicity which is based on color changes during the reduction of xtt. The xtt powder is slightly yellow and the formazan derivatives are orange.

In this study, we used xtt method to evaluate the cytotoxicity.

In brief, after trypsinization and detaching the cells from flask, resulted cells were cultivated in a flat 96-well culture plate at a density of 10⁴ cells/well in DMEM and cultured in incubator (37°C in 5% CO₂) to attach.

After 24hr, the medium was removed and cells were pretreated with DMEM without phenol red and FBS. Different concentrations of synthetic molecules (0.05, 0.1, 0.2 % (w/v)) were added to each well and then incubated for 24hr. Culture medium and solvents were used as controls.

After 24 hours XTT reagent solution (1mg/ml in pre warmed DMEM without phenol red and FBS) was prepared and to 1ml of this solution, PMS (N-methyl dibenzopyrazine methyl sulfate) in phosphate buffer saline (1.53mg/ml, 5µl) was added (reaction solution). PMS helps drive XTT reduction. Consequently, the reaction solution (50µl) were added to each well and after pipetting completely, the plate was incubated for 2-4 hours in incubator (37°C, 5% CO₂).

Finally, the absorbance of samples were measured using a photometer (ELISA reader, BioTek) at a wavelength of 450 nm.

3. Results and discussion

Pharmacologically, raloxifene is full antagonist of ER [25], so its efficacy to attach and block the receptor is more than other SERMs like tamoxifen . In order to conjugate raloxifene to chitosan, a linker which could bind to raloxifene from one side and bind to chitosan from another side is necessary. This modification can be achieved through silanization by using an appropriate

organosilane agent. One of the most widely applied organo-functional alkoxysilanes is (3-GPTMS). The epoxide functional groups are suitable for covalent binding formation with appropriate functional groups such as hydroxyl and amino groups. The O-C and N-C bonds are extremely stable; so that, raloxifene which contains the phenolic OH, as the only reactive group, can react with linker. Finally the epoxy group can undergo reaction with hydroxyl and amino groups of chitosan (scheme 1). In this study, raloxifene-chitosan conjugate were prepared by six different methods as shown in table1.

3.1. Characterization

3.1.1 FTIR studies

FT-IR was used to monitor the structural changes caused by the chemical modification of chitosan. The obtained infrared spectra are displayed in Figs.1-3.

The IR spectrum (Fig.1a) of unmodified low molecular weight chitosan (**1**) presented the expected characteristic bands as follows: a strong broad absorption band at 3423 cm^{-1} due to overlapping OH and amine N-H symmetrical stretching vibrations [26, 27]; a peak at 2922 cm^{-1} due to C-H stretching vibration [26]; peaks at 1646 and 1564 cm^{-1} due to -C=O stretching (amide I) and NH stretching (amide II) respectively [26, 28], amide III peak (CH_3 in amide group) at 1328 cm^{-1} (C-N stretching) and -CH_3 symmetrical angular deformation at 1380 cm^{-1} [26]; the absorption bands at 1154 cm^{-1} corresponding to the anti-symmetric stretching of C-O-C bridge, and 1058 cm^{-1} due to the skeletal vibrations involving the C-O stretching.

Figure 1b represents the spectra of trimethyl chitosan (**2**). The band centered at 1470 cm^{-1} in the spectrum of TMC, is attributed to the asymmetric angular deformation of C-H bonds of methyl groups. The characteristics bands due to the OH and amine N-H symmetrical stretching vibrations (3431 cm^{-1}), -C=O stretching (amide I) at 1634 cm^{-1} , C-O stretching between $1,100$ and $1,030\text{ cm}^{-1}$ do not change and confirm the lack of the introduction of an alkyl group at C-3 and C-6 in the chitosan.

Figure 2a, Shows the FTIR spectrum of raloxifene HCl (**3**). Characteristic peaks of raloxifene are observed at 2952 , 2853 , 2747 , 2691 and 2570 cm^{-1} but two peaks at 3205 and 3143 cm^{-1} which are related to two main functional groups, N-H and phenolic OH are the most important peaks [29]. It can be observed that there is a peak at 1641 cm^{-1} that can be assigned to C=O stretching

vibration. All the FTIR peaks were found to be in agreement with reported IR spectrum of raloxifene HCl.

Figure 2b depicts the infrared spectrum of 3-GPTMS (**4**). Si-OCH₃ characteristic band appear at 2841 cm⁻¹ (sharp band), 1194 and 1087 cm⁻¹ (strong bands). Si-alkoxy compounds have one or more strong bands in the 1000-1100 cm⁻¹ range which can be masked by C-O absorptions.

In the first step for creating conjugation between drug and polymer, raloxifene was reacted with linker in presence of triethylamine as a base. FTIR spectrum of the resulted linker-raloxifene conjugate is shown in Fig.2c. There are some peaks confirming raloxifene and linker reacted with each other; two peaks at 1651 and 1597 cm⁻¹ correspond to aromatic C=C stretching vibration which are absent in 3-GPTMS infrared spectrum and the main band due to Si-OPh stretching vibration at 1261 cm⁻¹ were observed.

FTIR spectra of synthetic compounds (**5**, **6**) are shown in Figure 3. In the product of run 1, dominant peaks are related to chitosan (Fig. 3a). To ensure that conjugation between drug and linker was done, linker/drug ratio was increased in run 2 but there was not significant changes in its FTIR spectra (Fig. 3b). In order to increase the effective interaction between raloxifene and linker, triethylamine was added in run 3 and LMW CS was also used to improve the solubility. FTIR spectrum of run 3, (Fig. 3c) showed that the intensity of OH stretching band is less than run1 and 2. In run 4, (Fig. 3d) the amount of triethylamine was tripled, but the FTIR spectrum wasn't shown very different results. To achieve the better solubility TMC was used instead of chitosan in run5. Characteristic peaks of chitosan and raloxifene can be seen in its FTIR spectrum (Fig. 3e). In 3402 cm⁻¹, the peak is due to the stretching vibration of O-H that is overlapped to the stretching vibration of N-H. The band at 2930 cm⁻¹ is due to C-H stretching vibration which is overlapped to raloxifene peak (2946 cm⁻¹). The peaks at 1626 cm⁻¹ and 1596 cm⁻¹, are related to amide I and II respectively which are overlapped to C=O stretching and C=C stretching of raloxifene.

In run 6, the Excess amount of linker-raloxifene was used to ensure that raloxifene and chitosan were totally conjugate via linker. IR spectrum of run 6, (Fig. 3f) indicates that the characteristic peaks of raloxifene and TMC are available. So it can be concluded that raloxifene-linker reacted with TMC successfully.

3.1.2. Thermogravimetric analysis (TGA)

TGA is a useful technique, to investigate the percentage of water uptake and degradation of polymers, etc. [26]. The TGA curves of LMW CS, raloxifene and final products of run 3 and 6, are shown in Figure 4.

In TGA thermogram of LMW chitosan (Fig.4), two steps of degradation are observed. Initial degradation exhibited 6.66 wt% weight loss and this degradation is occurred between 46 °C and 94 °C due to dehydration [26, 27]. Decomposition of chitosan starts at 257 °C and degradation continued up to 362 °C which the weight loss is 87.94 wt%. Thermal disintegration of glucosamine residues are responsible for the second degradation of chitosan [27].

Raloxifene thermogram indicates that there is no weight loss in the first stage; consequently, there was no absorption of water. Two steps of degradation due to the decomposition of etheric bonds and separation of aliphatic part of backbone are observed. The initial decomposition starts at 298.24 °C up to 351.68, exhibited 65.72% weight loss. The second stage of degradation is occurred at 508.6 °C and completed at 588.38 °C which the weight loss is 29.52 wt%.

Figure 6 represents TGA thermogram of synthetic molecule containing linker, drug and LMW chitosan (resulted from run3) as well. The pattern of this thermogram is similar to chitosan. Three degradation steps are observed. The first degradation is from 43.94 °C to 75.16 °C with 6.31% weight loss which is similar to initial degradation of LMW chitosan due to dehydration. The second step is occurred from 254.36 °C to 317.37 °C which 53.31 wt% weight loss is occurred. This step can be associated with the first degradation of raloxifene and the second degradation of LMW chitosan (Fig. 4).

The last degradation is from 475 °C to 570 °C with 35.81% weight loss. This step seems to be associated with second decomposition of raloxifene. Since the TGA thermogram of run 3, shows the same pattern as raloxifene and LMW chitosan, it can be concluded that the conjugation of LMW chitosan, linker and raloxifene was done successfully.

Similar to LMW chitosan, in TGA curve of TMC, two degradation steps are observed. Initial degradation exhibited 2.7 wt% weight loss and it is occurred between 50 °C and 126 °C due to dehydration [30]. Degradation of TMC starts at about 201 °C and continued up to 274 °C which 30.19 wt% weight loss is occurred.

TGA thermogram of synthesized compound in run 6 is interesting. Dehydration step is not observable because there was not absorbed water. Moreover, there is only one step degradation

from 231.05 °C to 325.77 °C with 61.15% weight loss. The start point of this decomposition is between the main decomposition start points of raloxifene (298 °C) and TMC (201 °C) approximately. This pattern is observed in case of decomposition end points (raloxifene: 351.68 °C, TMC: 274 °C and run 6: 325.77 °C).

Based on these findings, it can be concluded that conjugation of raloxifene to TMC resulted in these rational changes in decomposition points of raloxifene-TMC compared with pure TMC.

3.2. Physical properties assessment

In order to further examinations and comparing of physical properties of resulted molecules with their basic chitosan backbone, their melting/ glass transition temperatures and solubilities (as two important parameters which related to chemical structure) were evaluated.

3.2.1. Glass transition (T_g) and Melting temperature(T_m) Determination

T_g of synthetic molecules and chitosan and also T_m of raloxifene were determined and results are shown in Table 3. T_g for MMW chitosan, run 1 and run 2; were 250, 255 and 239 respectively. Since, T_g is one of the characteristic properties of material, different T_gs of synthetic molecules compared with chitosan and T_m of raloxifene, confirm structure change in the resulted products. Therefore it can be concluded that, chitosan and raloxifene are conjugated and made a new molecule. This pattern is observed for all the other runs.

3.2.2. Solubility

Solubility of raloxifene in organic solvents such as DMF and DMSO is 10 mg/ml and it is slightly soluble in water (0.1 mg/ml) [31]. Chitosan and TMC are soluble in acetic acid 1% (v/v) and water respectively. The solubility of synthetic molecules in these solvents was evaluated according to Table 2. The synthesized products were completely insoluble in DMSO, DMF, water and acetic acid. So, the solvent mixtures in different ratios were examined but the synthetic molecules didn't dissolved. Consequently, it can be concluded that resulted structures are different from raloxifene and chitosan.

3.3. Cytotoxicity studies:

Finally, two different molecules produced during run 3 and 6, which showed better results in other analysis, subjected to cytotoxicity assay using XTT method.

Results obtained from cytotoxicity assay (Fig. 5), indicated that there are no significant decrease in the cell viability after 24 hours exposure to chitosan-raloxifene resulted from run 3 in compare to LMW chitosan as a control with a cell viability of 100%. These results are similar to what obtained from run 6, in compare to TMC. For the chitosan derivatives used as drug /gene carriers, the low cytotoxicity is an important feature [32]. In this study, chitosan-raloxifene produced in both runs displayed low cytotoxicity (cell viability $\geq$ 80%) against mcf-7 cells in all polymer concentrations.

Conclusion:

In this study, conjugation between raloxifene and chitosan, as a novel chitosan derivative, was performed using different methods. FTIR and TGA analyses confirmed the formation of appropriate conjugates. Cytotoxicity assay of resulted molecules showed desire cell viability after 24 hours exposure. Therefore, it can be concluded that raloxifene- chitosan conjugate, as a new chitosan derivative, has a potential ability for using in targeted gene/drug delivery purposes. This new chitosan derivative is now under examination as a targeting nanoparticulate agent for anti cancer drug/gene delivery to breast cancer in another study in our laboratory.

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Reviewer #1 comments:

1.Reviewer's comment: Fig. 2c shows FTIR spectrum of the resulted linker-raloxifene conjugate. But the section 2. 2. 1 and 2. 2. 2 didn't describe the isolation of linker-raloxifene conjugate from its solution.

Authors' response:

We agree that this is an important area that requires further discussion. The product of first step (linker-raloxifene conjugate) was a yellow color precipitate which was separated from the reaction mixture and washed thoroughly with methanol for eliminating the excess amount of epoxy linker. The FT-IR spectrum of this conjugate was recorded and compared with the FT-IR spectra of raloxifene and 3-GPTMS to confirm the conjugation. In accordance with the referees' wishes, we have now added more explanation about of linker-raloxifene conjugate isolation from its solution (Experimental, section 2.2.1, (Experimental, section 2.2.1, page 4, line 108-110).

2.Reviewer's comment: How many is the molecular weight of chitosan (MMW and LMW)?

Authors' response: The Molecular weight of MMW and LMW are 127 KDa and 70 KDa respectively, which are now mentioned in the materials section (Materials and methods, Section 2.1, Page 4, line 90-91).

3.Reviewer's comment: Trimethyl chitosan (TMC) is one kind of chitosan derivative without amino group. Please indicate graphically the linking site at TMC molecule.

Authors' response: We support the referee's assertion but one important point which is not mentioned in the manuscript is the degree of quaternization of chitosan. Based on ¹H-Nuclear magnetic resonance spectra, the degree of quaternization of TMC was 30%, so still there are enough amino groups to react with epoxy linker, also hydroxyl groups are reactive against epoxide ring. More explanations were added in the manuscript (Materials and methods, Section 2.1, page 4, line 91). A figure indicating the structure of this conjugate was added to the main text (Results and discussion, Section 3, page 7, line 181).

4.Reviewer's comment: TGA is a really useful technique, to investigate the percentage of water uptake and degradation of polymers, etc., but is seldom used to characterize the structure of polymer. It is difficult to differentiate the decomposition sites (at chitosan, TMC, linker, raloxifene or their linking bonds?) of raloxifene-chitosan (or TMC) from its TGA curve.

Authors' response:

The view point of respected reviewer is true. But there are many studies in the literature which have utilized TGA method for evaluating of possible changes at the molecular structures especially in case of polymers [1-4]. Adding the new chemical groups or molecules to the polymeric backbone can change its response to the temperature. By increasing the temperature step by step, after leaving the water molecules, other molecules leave the polymer and make changes in the TGA curve. So, the TGA curves of synthetic conjugates are different from naked polymer.

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5.Reviewer's comment: Please explain using MCF-7 (a human breast cancer cell line) with XTT method.

Authors' response: As we mentioned at the manuscript (introduction session, page 4, lines 84-86) the ability of this conjugate for drug delivery to cancerous cells via estrogen receptors is under evaluation during another study in our lab. As you know MCF-7 cell line has expressed estrogen receptors on its surface. So, it is necessary to evaluate the toxicity of this new chitosan derivative on the cell line which it is suitable for our system. In this view MCF-7 is a good candidate. Moreover, according to the literature, to evaluate the ER positive breast cancer, MCF-7 cell line is used [5,6].

[5] Richard M. Neve, Kwei Chin, Jane Fridlyand, Jennifer Yeh, Frederick L. Baehner, Tea Fevr, Laura Clark, Nora Bayani, Jean-Philippe Coppe, Frances Tong, Terry Speed, Paul T. Spellman, Sandy DeVries, Anna Lapuk, Nick J. Wang, Wen-Lin Kuo, Jackie L. Stilwell, Daniel Pinkel, Donna G. Albertson, Frederic M. Waldman, Frank McCormick, Robert B. Dickson, Michael D.

Johnson, Marc Lippman, Stephen Ethier, Adi Gazdar, and Joe W. Gray. A collection of breast cancer cell lines for the study of functionally distinct cancer subtypes. *CANCER CELL* 2006, 10: 515.

[6] Deborah L Holliday, Valerie Speirs , Choosing the right cell line for breast cancer research. *Breast Cancer Research* 2011, 13:215.

Figure legends

Figure.1: IR Spectra of a) LMW CS b) TMC

Figure 2: IR Spectra of raloxifene

Figure 3: IR Spectra of linker

Figure 4: IR Spectra of linker-raloxifene

Figure 5: IR spectra of synthesized compounds: a) run 1, b) run 2 c) run 3, d) run 4, e) run 5, f) run 6

Figure 6: TGA thermogram of chitosan (LMW), TMC , raloxifene, run 3, and run 6

Figure 7: cell viability (XTT method mcf-7 cell line, 24 hours exposure)

Scheme 1: structural formula of **1**) low molecular weight chitosan; **2**) Trimethyl chitosan

3) raloxifene; **4**) 3-(Glycidyloxypropyl)trimethoxysilane; **5**) synthetic molecule in runs1-4,

6) synthetic molecule in run 5,6

Figure(s)

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6

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Table1: Runs components and their amounts

Run	Linker/drug (mol/mol)	Et ₃ N (μ l)	Chitosan
1	1	-	MMW
2	2	-	MMW
3	1	20	LMW
4	1	60	LMW
5	1	20	TMC
6	Excess amount of linker	20	TMC

Table2: solvents used for solubility test.

solvent	Solvents ratio
DMF	-
DMSO	-
acetic acid 1% (v/v)	-
DMSO/DMF	1:1
	1:2
	2:1
DMSO/ acetic acid 1%(v/v)	1:1
	1:2
	2:1
DMSO / acetic acid 10% (v/v)	1:1
	1:2
	2:1
DMSO/ water	1:1
	1:2
	2:1
Trifluoroacetic acid (TFA) 1%(v/v)	-
Water / TFA	1:1
	1:2
	2:1
DMSO/ TFA 1%(v/v)	1:1
	1:2
	2:1

Table3: melting/ glass transition temperature

Samples	Tm or Tg °C
Chitosan (MMW)	250
Chitosan (LMW)	229
TMC	219
raloxifene	264
run 1	255
run 2	239
run 3	233
run 4	246
run 5	243
run 6	252