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THESIS ASSIGNMENT

Characterization of protein-polymer conjugates via thermogravimetric analysis (TGA) and differential scanning calorimetry (DCS)

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Abbreviations

- **TA:** Thermal Analysis
- **DSC:** Differential Scanning Calorimetry
- **TGA**: ThermoGravimetric Analysis
- **TG:** ThermoGravimetry
- **DTA:** Differential Thermal Analysis
- **T_m:** Melting Point Transition
- **T_g:** Glass Transition Temperature
- **T_c:** Crystalline Temperature
- **ITC:** Isothermal Titration Calorimetry
- **BSA:** Bovine Serum Albumin
- **BSA-Br**: Bovine Serum Albumin Initiator
- **DTG:** Derivative Thermogravimetric Analysis
- **DDTG**: Second Derivative Thermogravimetric Analysis
- **PS**: Polystyrene
- **PMMA**: Polymethylmethacrylate
- **PPgA**: Poly(propargyl acrylate)

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Abstract

The present thesis aims to characterize different types of protein-polymer conjugate samples by two main methods of thermal analysis. These two methods are Thermogravimetric analysis (TGA) and Differential Scanning Calorimetry (DSC). The first method examines the changes in the mass of protein-polymer conjugate samples as a function of temperature, while the second method examines the physical properties of these samples as a function of temperature. In this thesis, a bibliographical outlook will summarize the principles of these methods and their applications in the characterization of protein-polymer conjugates. In the experimental part, the characterization of a variety of protein-polymer conjugates synthesized in the laboratory of Synthetic Biomaterials will be analysed. It is important to mention that this thesis is mainly bibliographical due to COVID restrictions. However, I was trained in both TGA and DSC analysis and performed a series of measurements. The TGA and DSC data for most of the samples that are analysed in this thesis were provided by members of the laboratory of Synthetic Biomaterials.

Chapter 1: Theoretical Background

1.1 Introduction

Thermal analysis (TA) is a branch of materials science where selected samples are introduced in specific temperature programs in order for their properties and physicochemical parameters to be studied, as a function of temperature or time.[1] Thermal analysis, apart from detecting and measuring the physical properties of materials, can also shed light on their mechanical and thermal history. As a result, new and alternative processes can be designed and applied not only for the industrial production of these materials, but also for the evaluation of their lifetime in different environments.[2] TA takes advantage of several techniques used in a plethora of studies and experiments. ThermoGravimetric Analysis (TGA) and Differential Scanning Calorimetry (DSC), being the two most frequently used TA methods, will be described in detail in this thesis.[3]

ThermoGravimetric Analysis was first used in order for the mass change of a measurement to be explained.[4] However, the first data of TGA were evaluated for kinetic purposes by Kujirai and Akahira, who studied the prediction of the thermal life of materials *via* the kinetic analysis of their thermal deterioration.[1] After the Second War World until today, significant improvement has been done concerning the TGA technique, which is widely used in polymer characterization.[4] In particular, TGA is a quantitative analytical technique which is used for the measurement of the polymer mass as a function of time or temperature, under controlled atmosphere.[3] TGA includes isothermal holds, cooling, heating or a combination of all these parameters, with the temperature which can be adjusted on the highest range of 1600 °C.[5,6] Furthermore, the controlled atmosphere is achieved by purge gases that are present at a TGA setup. This controlled atmosphere, which is mainly air or oxygen, oxidizes

metals and organic materials.[4,7] The purge gases can also be argon, helium or nitrogen, which do not react with the sample, enabling to perform experiments even when the mass of the sample changes more easily.[5]

The second most commonly used technique, DSC, was developed at the beginning of the sixties and up to date, various applications have been reported in polymer science.[3] DSC is an analytical technique that provides information about the physical properties of a sample such as, crystalline or amorphous nature.[8] During a DSC experiment, the sample is heated to a controlled temperature in which the heat flow rate difference is measured as a function of temperature.[8] This technique has been improved over time and presents significant results in high sensitivity instruments, demonstrating DSC as an accurate method. Importantly, DSC has been applied for the investigation of the thermodynamic properties of various pharmaceutical products, such as, proteins, biopolymers, lipid carriers and peptides, benefiting both organic and inorganic chemistry. [9-11] It is worth mentioning that DSC and TGA are extremely useful methods when only a limited amount of sample is available and more specifically few milligrams. To sum up, when TGA is coupled with DSC, changes between heat flow and the sample mass are observed. Interestingly, these changes are quite a useful tool in the detection of successful reactions since mass loss or gain is achieved.[12]

Together with TGA and DSC, some other important thermal analysis techniques are presented in Table 1.1 along with their measures.

The diverse general methods of TA indicate its versatility.[4] As mentioned before, temperature is the most important parameter of TA methods. Therefore, in many cases, the temperature-dependent changes in materials properties can determine a phase as clearly as its chemical formula or structure.[1] However, the TA

methods do not represent a kind of analysis of the structure, but instead determine properties.[1] For example if we examine a crystal structure material, these thermal methods allow their interpretation.[1]

Table 1.1: General methods and techniques of thermal analysis.[2]

General Method	Acronym	Measured Property
Differential Scanning Calorimetry	DSC	ΔT, differential power input
ThermoGravimetry or ThermoGravimetric Analysis	TG or TGA	Mass
Differential Thermal Analysis	DTA	ΔT
ThermoMechanical Analysis, ThermoDilatometry	TMA, TD	Length or volume
Dynamic Mechanical Analysis	DMA	Viscoelastic properties
Dielectric Analysis	DEA	Dielectric properties
Micro/Nano-Thermal Analysis	μ/n-TA	Penetration, ΔT

1.2 Short history of Thermal Analysis

Thermal analysis has a history of at least 13 decades.[4] Since it was first used, thermal analysis techniques have been developed and have found applications in different fields such as, metals, minerals, electronic materials, inorganic substances, ceramics, organic substances, food, pharmaceuticals, biological organisms and polymers.[4]

In 1743, a thermal analysis study was first reported by Antoine Laurent de Lavoisier, who observed a change in the mass of a sample upon oxidation.[13]

In 1887, Le Chatelier performed analysis experiments studying samples of clay minerals.[14,15] Le Chatelier used the heating-curve method and recorded the heating-curve utilizing a photographic plate, a light chopper and a galvanometer. These measurements resulted in strips that were printed on the photographic plate and the interval of these strips was correlated to the heating rate.

A few years later in 1899, Roberts-Austen performed the first differential temperature measurements resulting in the development of Differential Thermal Analysis (DTA).[16] This technique detects the temperature difference after the introduction of a thermally inert reference material subjected to the same heating or cooling program as the sample.

About 16 years later, in 1915, Honda discovered the measurement of mass change using temperature thermogravimetry (TG).[17] TG overcame the limitations of DTA, since the physical and chemical transformations observed by mass changes of the samples could be achieved at high temperatures without cooling However, until then at least two persons were needed to carry out the measurements lasting long periods of time without intermission, making thermal analysis a tedious and hard work.

After the War World 2, the technology of thermal analysis analytical equipment developed in terms of control and automatic recording. This automatic equipment became available mainly after 1950. Notably, in Japan there were three fully automated DTA apparatuses at the end of 1950s. At the beginning of the 1960s, automated thermobalances and power-compensated differential scanning

calorimetry (PC-DSC) were also commercially available. The automation led to a developing period of thermal analysis between 1960 and 1970. [18]

Up to now, the thermal analysis equipment has been formulated to consist of a furnace, a temperature controller, a transducer to measure the sample physical properties and a recorder for the output signal to be recorded as a function of the temperature or time, with a constant rate of heating or cooling in order for the temperature to be controlled.

One issue that has been surpassed nowadays, is the need for a large amount of the sample mass during the thermal analysis experiments, as the starting sample mass that was originally used at TA techniques was of the order of a gram or more. Currently, thermal analysis equipment necessitates use of a sample mass in the order of 10 mg or less.[4] Importantly, even smaller samples have been measured at TA apparatuses, such as in the case of pharmaceuticals substances. This development has led to a beneficial and improved temperature resolution of the sample, reducing the response time of the apparatuses.[4]

Nowadays, there is great interest concerning the applications of TA techniques in materials science, due to the thermal behaviour of materials in the solid state, while in the liquid state, special variations of TA methods are needed.[19] TA techniques are mainly used for heat capacity measurement, kinetic analysis and purity determination.[19] There is an increasing interest to measure for organic samples, as they are used as precursors to pharmaceuticals and they present thermal properties that are mainly used in quality control procedures.[19] A variety of common pharmaceuticals are evaluated using mainly the DSC method, which allows the estimation of the pharmaceuticals' activity and the 'lifetime', as well as the purity

definition.[19] Concerning bulk materials, such as polymers, glass transitions and polymer blends have been studied using DSC.[19] Finally, biological materials such as edible oils, fats and chocolate have also been studied using TA techniques.[19] The aforementioned TA techniques apart from the properties mentioned above, also study many chemical or physical in nature phenomena such as, glass formation, melting, electrical properties of solids, heat capacity, dehydration, sublimation, swelling, sintering and cracking, thermal expansion, structural defects, oxygen content, reaction kinetics, vaporization, decomposition, surface morphology, linear and bulk deformations.[3]

1.3 Differential Scanning Calorimetry (DSC)

1.3.1 General Information

Differential scanning calorimetry (DSC) was first developed in the early 1960s whereas the first DSC apparatus belongs to the Perkin Elmer Company.[11] DSC measures the thermodynamic characteristics of materials and more specifically detects the properties of thermally induced transitions with various applications in a wide range of scientific fields including polymers, pharmaceuticals, organic and inorganic compounds.[20-23] A significant advantage of DSC is the independence of the DSC signals from experimental conditions, allowing DSC experiments fora greater precision.[24] In addition, the differential heating abilities of DSC enabled scientists to compare the energy difference between a reference material and the sample, without being affected by extraneous factors or solvents.[25]

Concerning the DSC system, heat is being absorbed or released in order to maintain the temperature of the sample and the reference sample similar. At the same time, the DSC system is subjected to processes, such as cooling or heating, in

a specific inert atmosphere (usually nitrogen) or oxidizing atmosphere (oxygen or air).[26] It is well known that heat is an extensive thermodynamic quantity transferred spontaneously from hot to cold systems. Therefore, the value of the heat is proportional to the mass of the system and constitutes the most important quantity in DSC system.[26]

More specifically DSC is used for the estimation of the thermal properties of materials like polymers and bioconjugates including melting point, crystallization and glass transition. Significantly, the high caloric sensitivity of DSC renders in as the appropriate tool for investigating structure transitions in polymeric samples.[11]

1.3.2 Theoretical and experimental background

As mentioned before, materials present several properties which depend on temperature. Enthalpy, being one of them, can be calculated via DSC. Heat absorption from samples (endothermic variation) increases the enthalpy of the system, while heat release (exothermic variation) decreases the enthalpy, which is proportional to the heating rate (°C/min), the weight of the sample (w) and the variation of heat capacity.[11]

When a thermodynamic system is being studied, a constant volume is needed. When the system is polymeric, a constant pressure is maintained, as polymers are liquids and solids which are almost incompressible.[27] Enthalpy (H) is estimated by an equation at a constant pressure, which is mentioned below:

$$H=U+p*V \quad \textbf{(1.1)}$$

where, p: pressure, V: volume and U: internal energy.[11]

According to equation (1.1), the enthalpy of the system increases as heat is being added to the system.[27] Moreover, practically there are enthalpy differences, like total absolute enthalpy cannot be directly calculated, but only when the heat capacity of the whole temperature range is given.[11] Therefore, the difference temperature between the reference and the sample, indicates the enthalpy change.[11]

Another thermodynamic property that depends on temperature is entropy. Entropy is described by an equation of the disorder of the system, which was introduced in 1865 by Clausius and is mentioned below:

$$\Delta S = S_B - S_A \geq \int_A^B \frac{\delta Q}{T} \quad \textbf{(1.2)}$$

where the equal (=) part of the sign refers to reversible processes, while the greater than (>) part of the sign refers to irreversible processes. Therefore, in the equal part of the equation (1.2), spontaneously irreversible processes would proceed if the entropy was increased in an isolated system. [11,27]

In calorimetry, the heat flow provides information about the energy of the sample. Heat flow is classified into three mechanisms, thermal radiation, conduction and convention.[8] Concerning the first one (thermal radiation), it occurs through a vacuum or a transparent medium. According to this mechanism, electromagnetic energy, by means of electromagnetic waves, is radiated from the surfaces of materials, especially when the material is solid.[28] The frequency and the intensity of thermal radiation energy depend on the increase of the temperature on the surface of materials.[28] The second form of energy (heat conduction), is the study of heat conduction between solid bodies in contact. In other words, molecules or atoms deliver to their neighbours one part of their vibrational energy.[29] Finally, the third

form (heat convection) is the transfer of heat from one place to another by the movement of fluids. In this case, heat is transferred from the surface of a solid material to a liquid or gas material and the opposite and is usually the dominant form of heat transfer in liquids and gases.[30]

In Differential Scanning Calorimetry, the specific heat (c_2) of a solution is calculated by the following equation:

$$c_2 = c_1 + 1/w_2(c-c_1) \quad \textbf{(1.3)}$$

where w_2: the weight fraction of the solute, c_1: the heat of the solvent and c: the heat of the solution. [8,23] DSC measures the value of ($c-c_1$) as the excess apparent specific heat (c_{ex}).[8,21] More specifically, heat capacity of the sample solution (c_s) can be explained by the following equation:

$$C_s = m_s x C_s^o \quad \textbf{(1.4)}$$

where C_s^o and m_s: the specific heat capacity and the mass of the sample respectively.[8,21] Also, for the heat capacity of the solvent (buffer) (c_b) the equation is similar:

$$C_b = m_b x C_b^o \quad \textbf{(1.5)}$$

where 'b' characterizes the buffer solution.[8]

According to a thermodynamic explanation of differential scanning calorimetry, the sample is undergoing phase transition, in which it changes phases at specific temperature or pressure.[11] The first classification of thermodynamic phase transition was reported by Ehrenfest in 1933, based on the behavior of the thermodynamic free energy as a function of other thermodynamic variables.[31] According to Ehrenfest phase transitions are divided into two broad categories (First

and Second Order). First-order phase transitions present a discontinuity in the first derivative of the free energy with respect to thermodynamic variables. Solid, liquid or gas transitions are categorized as first-order transitions as they involve a discontinuous change in density, which is the first derivative of the free energy with respect to pressure.[31] Second-order phase transitions are continuous in the first derivative of the free energy, but exhibit discontinuity in a second derivative of the free energy. Some examples for second-order transition are the superfluid transition of helium and the magnetic transition at the Curie point. In polymers, first-order phase transition such as melting of crystalline polymers is used.[31]

As mentioned before, DSC can fast and easily determine the glass transition, melting and crystallization temperatures.[1] In order to explain these temperature points, samples of amorphous, semi-crystalline and crystalline materials are taken.[32] First of all, the temperature which changes a crystalline solid to an isotropic liquid, is called the melting point (T_m). Melting point describes the edge of a specific curve in a DSC diagram, which is created by a sample of a low molecular mass and high purity.[32] In semi-crystalline polymers and materials with low purity and low molecular mass another temperature point (T_{mp}) is reported, which in the case of polymers is attributed to the maximum rate of the melting process.[11] Nevertheless melting point is defined as the temperature at which the most perfect crystallites melt and is determined as the highest temperature point of the melting endotherm.[32] The figure below (*Figure 1*), illustrated how the melting point can be found in a DSC curve with all the characteristics mentioned before.

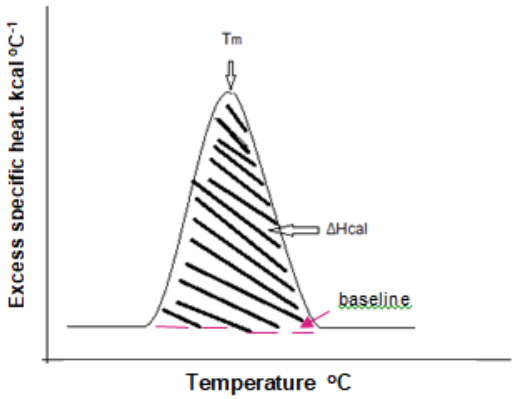


Figure 1: DSC endotherm curve showing the variation of excess specific heat with temperature during a two-state process.[8]

Sometimes multiple melting peaks are observed at a DCS diagram and can be explained in three possible ways.[11] Firstly, the multiple melting peaks can be attributed to many crystal forms of polymers that will melt and result in multiple endotherms.[11] Secondly, recrystallization can take place during melting procedure and thirdly the polymeric sample can be present during the whole melting procedure in crystalline form.[11]

Important thermal transitions apart from melting temperature (T_m) include the glass transition temperature (T_g) and the crystallization temperature (T_c). The glass transition temperature (T_g) of a material characterizes the range of temperatures over which glass transition occurs and it is always lower than the melting temperature (T_m).[32] In the case of polymers, conformational changes of segments become infinitely slow below the glass transition temperature. When a polymeric sample is heated up to the glass transition temperature, its state changes to liquid or elastic. Below this temperature the vibrational motion of chain segments is active and the glassy structure does not relax. [11]

The third thermal transition is the crystallization temperature (T_c), which changes an isotropic liquid to a crystalline solid upon cooling.[11] Crystallization describes the edge of a specific curve in DSC with the extrapolated baseline, created by a sample of low molecular mass and high purity.[11] It's worth to be mentioned that the melting point is almost always higher than the crystallization point because of supercooling.[11]

When a polymeric sample is characterized by DSC, different transitions are observed, depending to its structure. These transitions are presented with curves at a DSC diagram.[11] T_g is characteristic for the mobility of a polymer chain. This means that the limitation of rotational motion of the polymeric chain results in the increase of the T_g. On the other hand, when the chain is more mobile, decrease of T_g occurs.[11] In order to explain the glass transition temperature measurement at a DSC diagram, an informative figure (*Figure 2*) is given below.

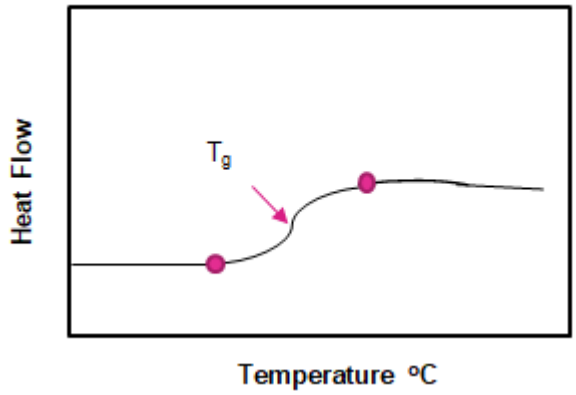


Figure 2: This DSC curve is an example of glass transition (T_g) of a polymer. The purple dots help the operator to find and calculate this temperature.[33]

DSC presents a crystallization transition curve when an amorphous material is transformed into a crystalline form.[33] This curve is shown in the figure below (*Figure 3*).

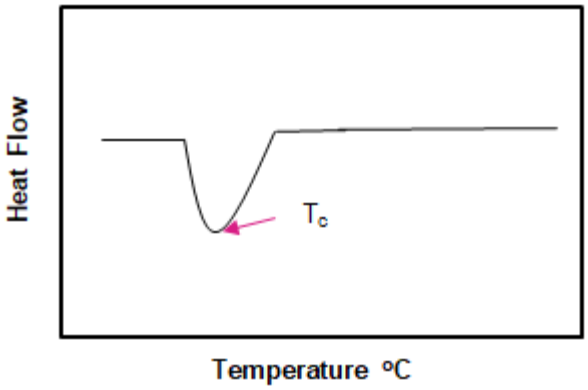


Figure 3: A DSC curve as an example of crystallization transition of a polymer.[33]

Crystallization of a polymer occurs at temperatures below the melting point T_m and above the glass transition point T_g. Above the glass transition point, i.e. as the temperature increases, the structure of the polymer chain changes, while below the glass transition temperature, the movement of molecular chains is frozen. Nevertheless, secondary crystallization can proceed even below T_g.[11] This kind of crystallization is called melt crystallization of polymers and is divided in two categories: (1) Isothermal crystallization: in which the polymer sample is first heated to above its melting temperature and then, it is kept at that constant temperature to fully melt out any existing crystals, (2) Non-isothermal melt crystallization: a process which is generally carried out under continuous cooling conditions leading to the crystallization of the polymeric sample.[11] Polymer crystallization that takes place above the glass transition temperature upon heating with prior cooling or permanence of the sample at temperatures below T_g is called cold crystallization process and can be both non-isothermal and isothermal.[11] This type of crystallization occurs at temperatures well below the melting region, far from equilibrium, and due to high nucleation density, it is generally fast.

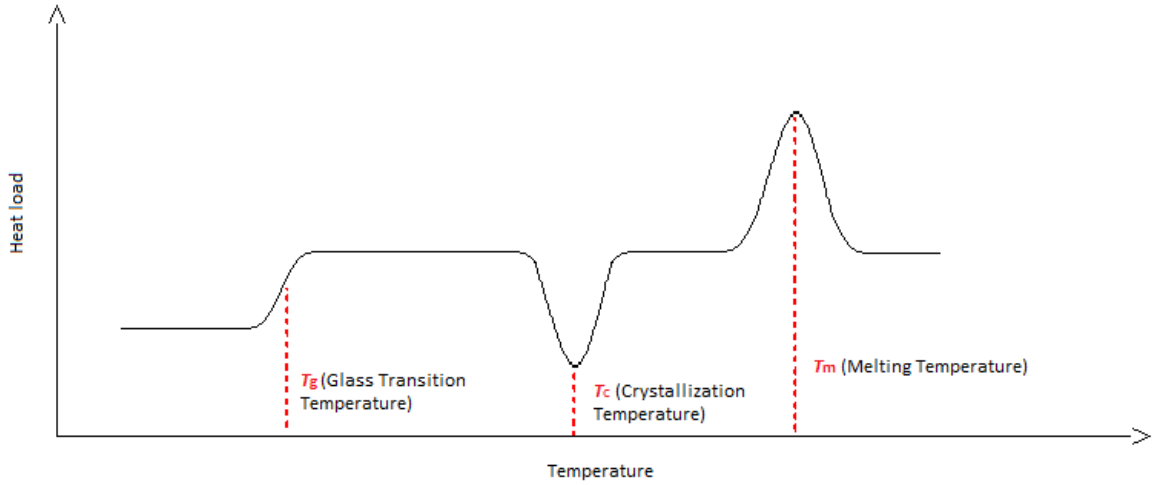


Figure 4: All transitions in one graph.[33]

For the experimental part of DSC that will be described in a forthcoming chapter, a Pyris Diamond DSC from Perkin Elmer will be used. Pyris Diamond DSC is a unique power compensation machine, which offers information about processes in material samples. It consists of two independent furnaces which include the sample and the reference sample both in a hermetically sealed aluminium pan (*Figure 5*). [11,34] These furnaces are heated from a temperature-controlled heat sink. This design allows the direct analysis of the measurement by calculating the heat flow out or into the sample. The characteristics of the Pyris Diamond DSC are high caloric accuracy, including a temperature range of -70°C to 730°C, low mass of the samples (<1g) and scanning rates of heating or cooling from 0.01 °C to 500°C/min.[11,34] Also the Diamond DSC comprises a gas supply tubing which provides the system with pure gas and dry nitrogen gas.[34]

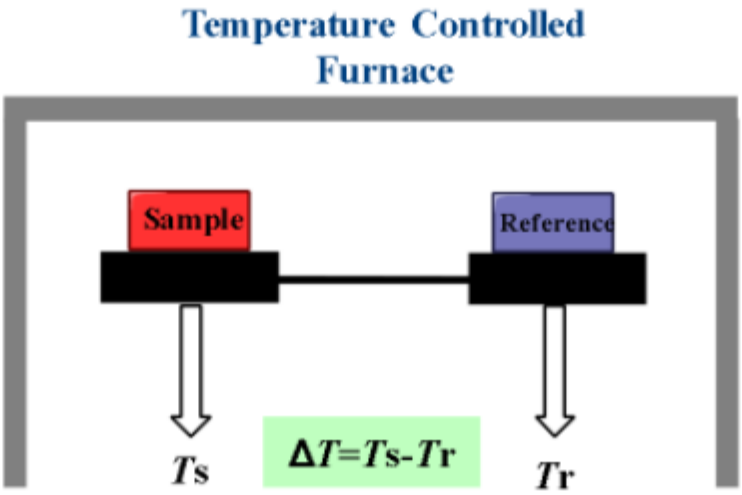


Figure 5: Representation of the furnaces, Ts: the temperature of the sample, Tr: the temperature of the reference sample.[34]

1.3.3 Applications in biomolecules

The mobility, stability and the structure of protein-based materials have been investigated using Thermal Analysis techniques, such as dynamic mechanical analysis (DMA), differential scanning calorimetry (DSC) and thermogravimetric analysis (TGA).[35]

Given that standard protein analytical methods are not always able to give information for chain architecture, Gavin *et al*. used DSC, among a series of analytical techniques, in order for the differences in multiple protein fractions of bovine blood to be analyzed.[36] DSC was used to measure changes in heat flow for a sample and a reference in order to detect protein denaturation. Absence of a denaturing peak in the DSC diagram of a blood meal sample (that is commonly analyzed for native proteins) was observed leading to the conclusion that extensive

denaturation of blood meal took place, suggesting the formation of a very different molecular arrangement very tightly packed.

DSC has also been applied in the determination of the affinity of drugs to receptors, enzymes, or transport proteins. For example, Sedov *et al*. used DSC to compare the ligand binding to the protein, observing the increase of the protein denaturation temperature, which leads to maximization of the peak in the DSC curves.[37] More specifically, the evaluation of quantitative parameters of binding such as stoichiometry, binding constant and enthalpy from the DSC curves took place. DSC studies showed that different concentration of a ligand can possibly affect and explain the binding constant of the ligand to a protein. According to the results of this study, DSC experiments on protein denaturation can be applied for the discrimination of substances binding, as well as the determination of the binding constants.

Aquasomes (AQ) are self-assembled nanostructures, commonly used for delivering bioactive molecules like proteins. Damera *et al*. reported AQ as an efficient dual drug delivery system that can release simultaneously the bioactive molecule and the hydrophobic drug.[38] The release ability of hydrophobic drugs was studied by investigating their binding interactions with BSA, using various techniques such as DSC. The DSC studies provided crucial information that BSA retained its structure even after the adsorption on AQ, since similar temperature range for thermal decomposition of BSA was observed before and after the adsorption on AQ. Similar binding interactions observed by DSC studies indicate that AQ/BSA can be used for sustained drug release.

Berberine (BBR) being a plant-derived isoquinoline alkaloid, has been reported to present pharmacological properties, such as anticancer activity. However, its use in clinical trials is prevented by the low water solubility and bioavailability. Solanki *et al*. overcame these limitations encapsulating BBR in BSA nanoparticles, which was confirmed by DSC studies.[39] More specifically, the endothermic peaks that are attributed to BSA or BBR nanoparticles were not observed in the DSC thermogram of BBR-BSA nanoparticles, which provided a new peak, indicating the successful encapsulation of BBR into BSA in amorphous form. DSC studies in combination with activity and stability studies suggested BBR-BSA nanoparticles as a novel tool for breast cancer treatment.

At the basis of these results the application of DSC in biomolecules provides information about the effect of the environment (e.g., ligands, temperature) on them and allows the quantification of the thermodynamic parameters to be achieved. In conclusion, DSC can provide structural and mechanistic information into protein function.

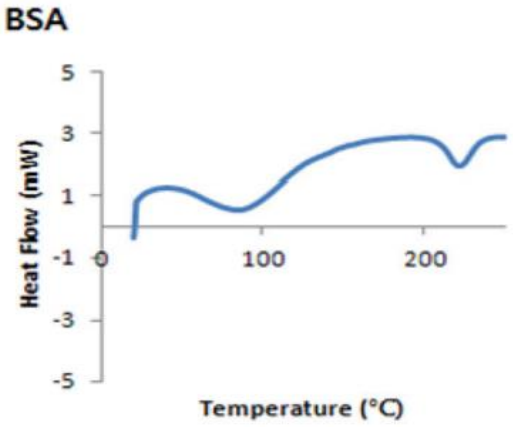


Figure 6: DSC thermograph of a native BSA sample. Reprinted by permission from Springer Nature: on behalf of Cancer Research UK: Springer Nature, Journal of

Pharmaceutical Investigation,[40] The effect of Eudragit type on BSA-loaded PLGA nanoparticles, Park, M.H. Baek, J.S. Lee, C.A. Kim, D.C. Cho, C.W .Copyright © 2014, The Korean Society of Pharmaceutical Sciences and Technology, 2014.

1.4 ThermoGravimetric Analysis (TGA)

1.4.1 General information

Thermogravimetric analysis (TGA), is a thermal analysis technique taking place under an inert atmosphere of argon or nitrogen and a controlled temperature variation program, where the mass of the sample being measured is provided as a function of time or temperature.[41] The first gravimetric experiment dates back to 27 BC and was referred to the measure of the mass change of a limestone which was calcined to lime and was reported by Vitruvius.[41]

Generally, TGA monitors the mass of a sample during decomposition and evaporation of the volatile compounds, where decrease of the mass is observed. A thermogravimetric analyser can also create inert atmosphere, alike to the DSC analyser, using argon, nitrogen or helium or oxidizing atmosphere such as oxygen and air.[3] In the case of organic or metal-based samples, oxidizing atmosphere has a tendency to combust and oxidize them respectively.[42,43] In contrary, inert atmosphere with nitrogen, helium or argon, under low temperature conditions does not affect (react with) the sample, unless the sample includes metals like Mg which reacts with nitrogen.[12] Since different oxidation states can complicate the mass analysis, inert atmosphere provides advantages in studies of thermal stability, as mass changes are more readily attributable. In addition to this, TGA gives maximum furnace temperature up to 1600°C and the optimum sample mass ranges from 1 mg to several grams.

In the case of polymeric samples, inert atmosphere is needed.[3] Mass loss is generally exhibited, but, under an oxidizing atmosphere mass gain could be observed prior to degradation of the sample, at slow heating rates.[3] Volatile components, such as residual solvents, oligomers, absorbed moisture or low molecular mass additives evaporate between 25°C and 300°C, while reaction byproducts, such as formaldehyde from the cure of phenolic and amino resins, between 100°C and 250°C, are characteristic of the mass loss.[3] Mass loss is also noticed from chain scission which produces volatile degradation products that require temperatures above 200°C but not more than 800°C to evaporate.[3] All these data are processes which need to be studied in order to gather information about thermal stability, extent cure and composition.[41]

To conclude, TGA coupled with DSC experiments, monitor changes in mass and heat flow, detecting a successful or unsuccessful reaction *via* the mass loss/gain, characterizing as well transitions like crystallization or melting point.[12]

1.4.2 Theoretical and experimental background

In TGA, sample analyses proceeds *via* a temperature program which includes cooling, isothermal holds, heating or a combination of these.[6] The TGA apparatus includes two precise micro balance arms. One has a counter pan on the top, without containing any sample and both are inside a furnace, which imparts controllers-thermo-balance arms and a temperature program.[3] In order to run an experiment, it is necessary to load the sample on a pan and place it on the top of the second micro balance arm.[44] After that, the furnace is closed and the TGA software interface displays different measurements.[44] Near the pan, there is a thermocouple

monitoring the sample temperature.[44] There is also a protective tube in order to isolate cooling coils and heating elements from the sample.[44] At 20-200 mL/min, a dynamic purge gas passes over the sample, which enters from a capillary tube and penetrates the sample pan.[44]This system configuration containing the sample holder, the gas entry and exit points and the furnace, prevents the mass flow signal from the noise and minimizes the turbulence near the sample pan.[41] Moreover, the flow stream in the top and side loading configurations is parallel to the cooling coils and the heating elements.[41]

As mentioned before, there is a counter pan inside the furnace which ensures the symmetrical electromagnetic balance and reacts as a counterweight with small dynamic range.[41] Unfortunately, without a counterweight, a greater electromagnetic force is required, in order for the balance sensitivity, which effects the sample pan, to be reduced.[41]

An upward force, produced on the top of the sample by the surrounding atmosphere (buoyancy phenomenon), affects the mass of the sample during a TGA experiment.[3] In other words, the increasing temperature and the inert atmosphere affect the balance.[3] Also, buoyancy effects are not counteracted by counter pans, due to the differences in turbulence and gas flow between the counter pans and the sample.[12] To address this problem, symmetrical TGAs have been designed, in order for buoyancy effects to be neutralized. These TGAs instead of having a counter pan, simply act as a counterweight. Counterweight's furnace uses a symmetrical gas flow into TGAs machines to create an electromagnetic optical balance with high sensitivity.[12] In conclusion, two identical furnaces are responsible to provide to both pans a symmetrical gas flow.[3,12]

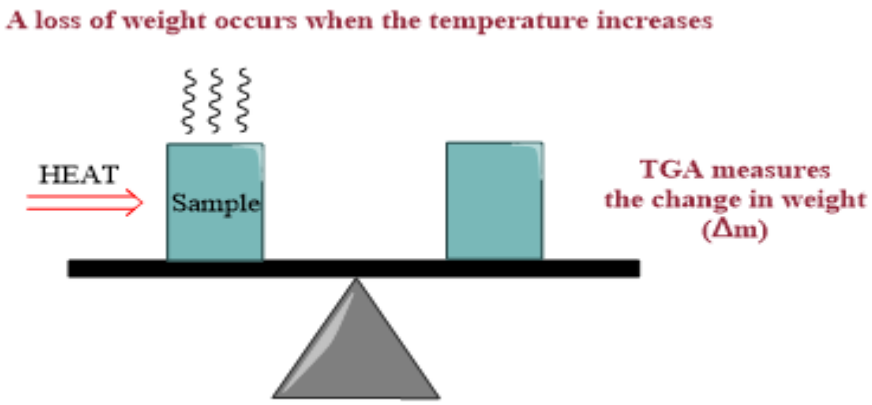


Figure 7: Representation of how heat passes through the sample into a TGA device.[3,12]

As has already mentioned, TGA is a characterization method, as thermograms provide information concerning the oxidative stability of the sample, the product lifetime, the moisture and volatiles content, the composition, the decomposition, the kinetics and the material thermal stability, properties that are unique for each compound.[45,46] More specifically, there are sections at TGA thermograms related to mass or temperature change.[3] The first mass loss observed in a TGA thermogram (Figure 8) is mainly attributed to solvents, physisorbed water, trapped gases and low molecular weight volatiles and is detected at temperatures from 150°C and under.[12] Additives, volatile decomposition products and chemisorbed water are responsible for the mass loss in temperatures between 150°C-250°C.[12] Between the onset and endset temperature, compounds begin to decompose in temperatures above 25°C. It is important to mention that in multi component systems there are reactions with intermediate steps, leading to multiple onset and endset temperatures.[12] Finally, the remaining material which includes non-volatile inorganic metals and ashes remain inside the sample pan in temperatures above the endset temperature.[12]

Except from the TGA thermogram mentioned above, we also receive through the appropriate software the first derivative (DTG) and the second derivative (DDTG) of the weight loss, as thermograms called Derivative Thermogravimetric Analysis.[47-49] These thermograms discriminate phenomena of multicomponent mixtures and identify inflection points at overlapping temperatures.[47-49] In case of overlapping peaks, the DDTG (onset temperatures) separate reactions more precisely as compared to the peaks of the DTG, which correspond to inflection points.[50] The temperatures that describe the TGA thermograms with their corresponding curves, are shown in Figure 8.

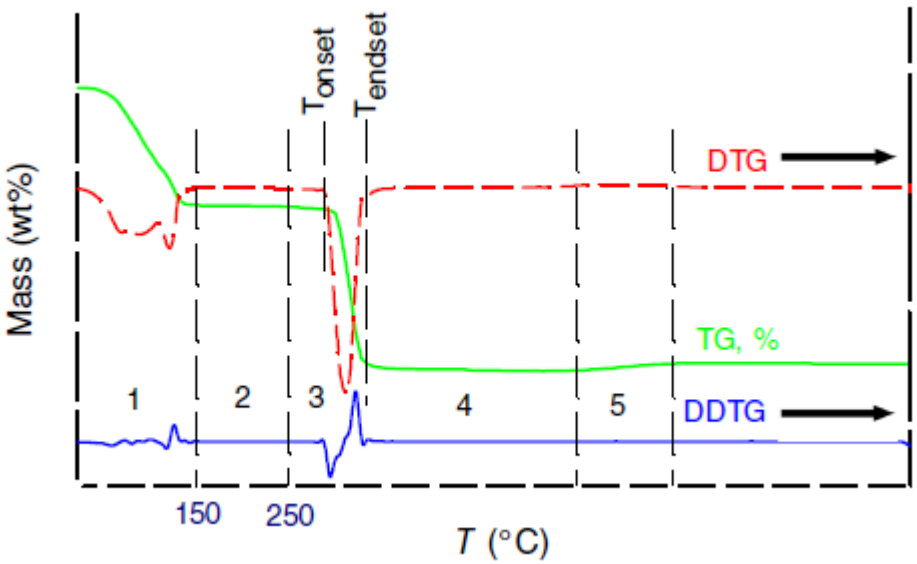


Figure 8: Example of TGA thermogram (green line), DTG thermogram (red line) and DDTG thermogram (blue line), with five possible statements of an experiment. Reprinted by permission from John Wiley and Sons Canadian Journal of Chemical Engineering, Experimental methods in chemical engineering: Thermogravimetric analysis—TGA, Saadatkhah, N., Garcia, A. C., Ackermann, S., Leclerc, P., Latifi, M., Samih, S., Patience, G. S., Chaouki, J, © 2019 Canadian Society for Chemical Engineering, 2019.[41]

1.4.3 Applications in biomolecules

As has already mentioned, ThermoGravimetric Analysis, like Differential Scanning Calorimetry, is a thermal analysis technique commonly used for the evaluation of the stability and mobility of polymers and protein-based materials.[35, 51-53]

For example, Tazhbayev et *al*. compared the synthesis of nanoparticles composed of BSA and HSA. The drug release profile of the products was studied by characterizing the products with thermal analysis techniques, including TGA.[54] More specifically, BSA and HSA protein nanoparticles were formed stabilized via the formation of intermolecular disulfide bridges when using urea and cysteine. Hydroxyurea was loaded in the nanoparticles through adsorption. A peak detected in the thermogram at 144.6°C is attributed to the endothermic peak of hydroxyurea. However, when the temperature was further increased an exothermic peak at 167.8°C was detected. The mass loss that was related with this temperature area was associated with the degradation of the drug, indicating the ability of these nanoparticles to be successfully used for drug release.

As has already been mentioned Damera et *al*. reported AQ as a dual drug delivery system that can release a bioactive molecule and a hydrophobic drug at the same time.[38] Apart from DSC, the release ability of the hydrophobic drugs was also studied by TGA. The weight loss curves of HAP, AQ, and AQ/BSA were measured. The HAP core presented a weight loss of about 4% below 500°C that was attributed to water loss. On the contrary, AQ, presented 10% weight loss that was observed at a temperature range of about 200-350°C. For AQ/BSA, about 20% weight loss was

observed, with the weight loss at 400-500°C to be attributed to cellobiose decomposition.[39]

The aforementioned study of Solanki *et al*. based on the encapsulating BBR (berberine) in BSA nanoparticles which was confirmed by thermal analysis studies,[39] also applied TGA in order to confirm the encapsulation of BBR in BSA. According to the TGA thermograms of pure BSA, pure BBR and BBR-BSA nanoparticles the total weight loss of BSA was 75%, while the weight loss of BBR was 50%. Significantly, the total weight loss of BBR-BSA nanoparticles was 81%. The increase of the total weight loss was attributed to the drug that was loaded in the BSA nanoparticles.

Recently Fragoso *et al* reported the preparation of carbon nanoonions (CNOs) which were used as supports to immobilize alkaline phosphatase, horseradish peroxidase, and glucose oxidase.[55] The CNOs were initially functionalized to bear carboxylic groups exposed on their surface and then coupled to the proteins using carbodiimide as coupling agent. The CNO–enzyme conjugates were characterized by Transmission Electron Microscopy and Raman spectroscopy. Interestingly, the TGA profiles of the CNO conjugates were found to be strongly dependent on the amount of immobilized enzyme which allowed to determine the degree of functionalization revealing a specific enzyme load of ~0.5 mg of protein per milligram of CNO as shown in Figure 9.

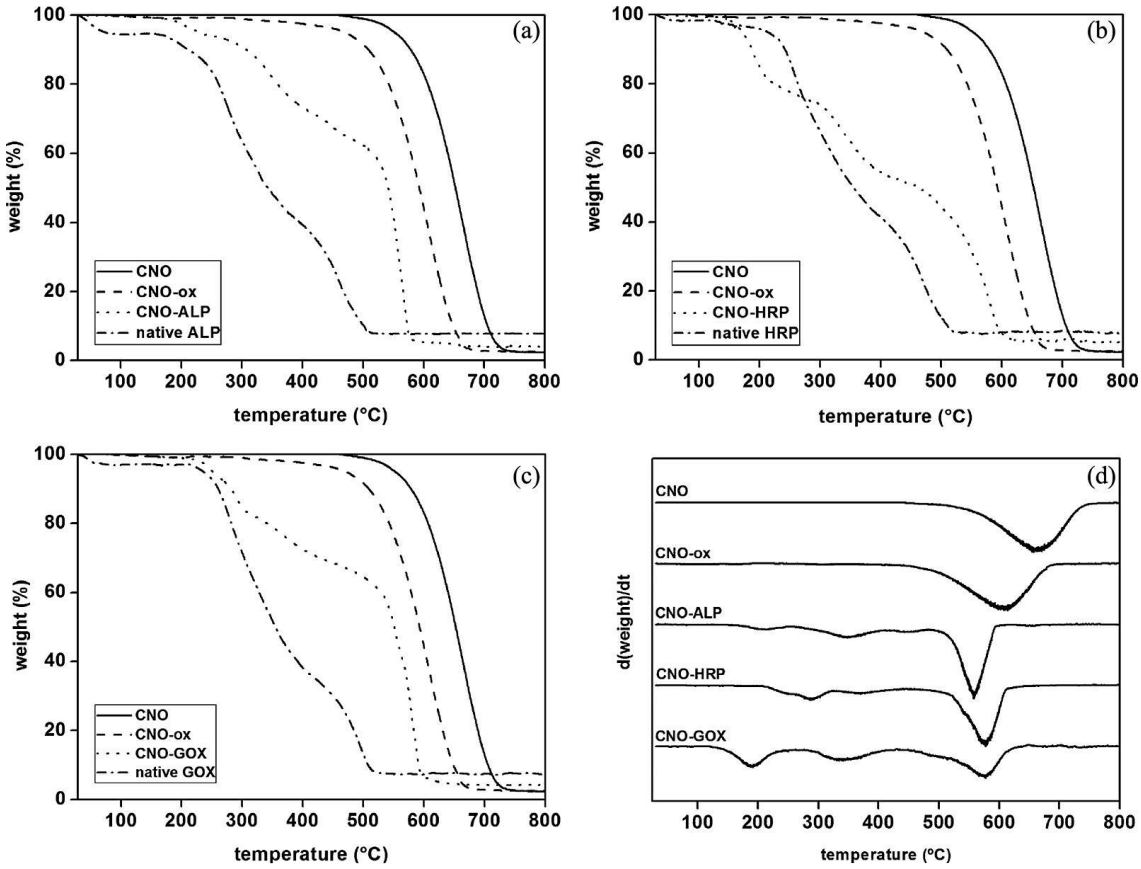


Figure 9: (a-c) Thermogravimetric analysis of CNOs, oxidized CNOs, and CNO–enzyme conjugates and (d) first derivative curves.[55]

Similarly, Theodorou *et al*. recently reported on the synthesis of protein-polymer conjugates via an oxygen tolerant photoinduced controlled radical polymerization approach.[56] Interestingly in this study also the TGA profiles of the bioconjugates were found to be strongly dependent on the degree of polymerization as shown in Figure 10.

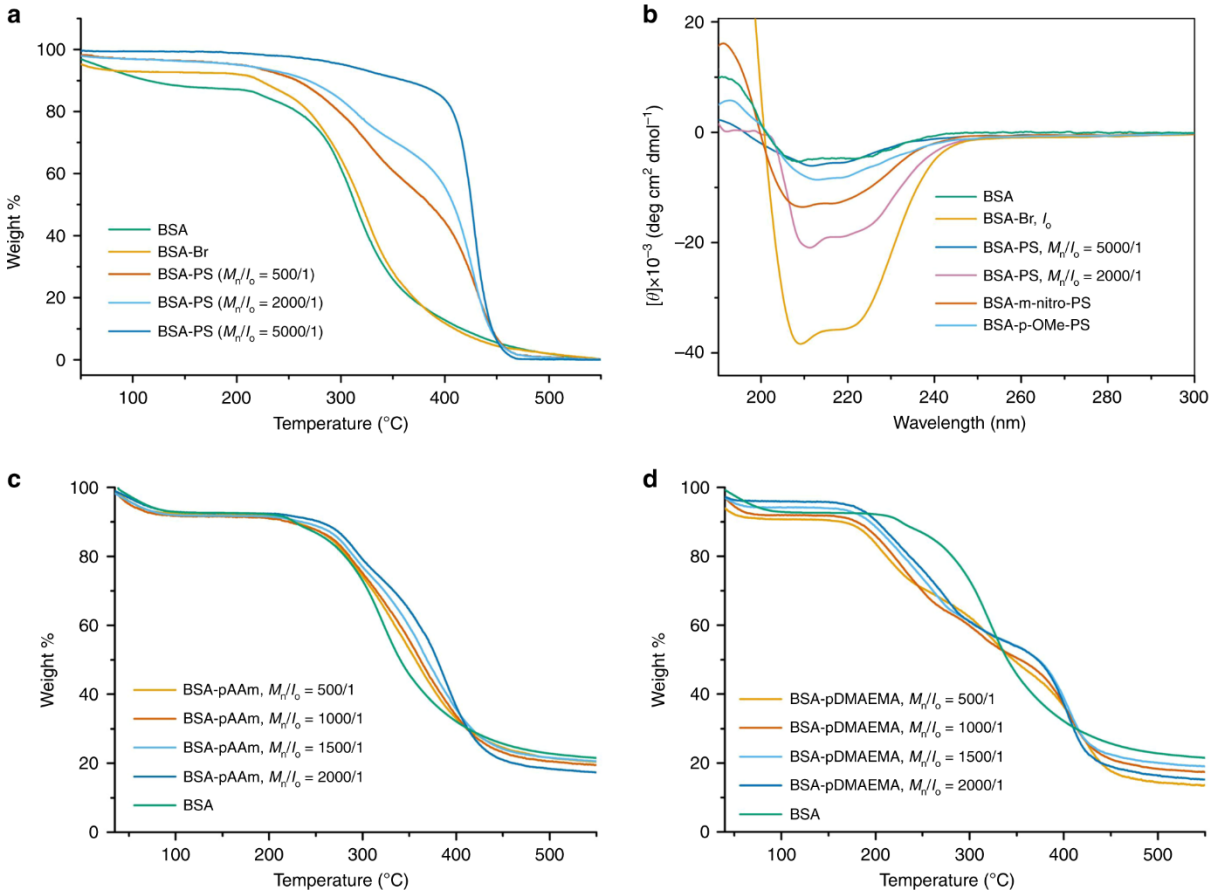


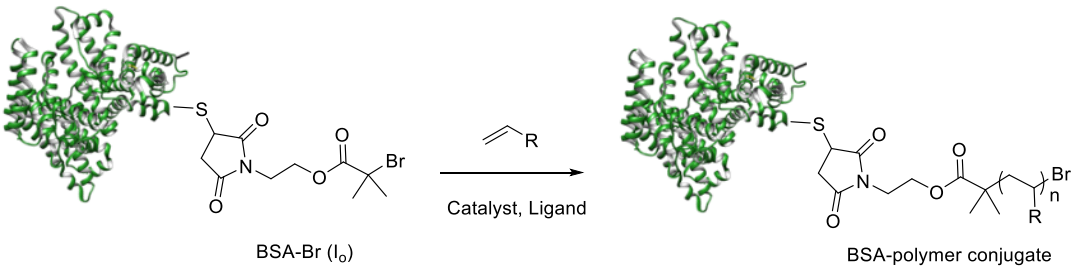
Figure 10: Thermograms of native BSA, the macroinitiator BSA-Br, and a) BSA-PS conjugates, c) BSA-PAAmm d) BSA-PDMAEMA. All measurements were performed in N_2 atmosphere.[56]

Taking these last examples as inspiration, the aim of this Thesis was to analyze TGA measurements of a larger variety of protein-polymer conjugates in order to extract further insight on the information which can be obtained through TA of protein bioconjugates.

Chapter 2: Aim of the thesis

The aim of the present thesis was the characterization of protein-polymer conjugates via thermal analysis and more specifically via TGA and –to a lesser extend- by DSC. All the samples were composed of BSA (as the protein part) covalently linked to a unique polymer. The samples differed in the chemical structure and molecular weight of the polymer moiety entailing different properties to the biohybrids and, in the preparation method.[57]

In summary, BSA-polymer conjugates were prepared via a grafting *from* approach, i.e., through an initial preparation of a biomacroinitiator (BSA-Br) followed by grafting monomers via controlled radical polymerization (Figure 11).



Scheme 1: Schematic representation of the general approach followed for the synthesis of BSA-polymer conjugates.

The bioconjugates studied in this Thesis are mostly amphiphilic as most of the grafted polymers are hydrophobic in nature. The inherent amphiphilicity of such biohybrids renders characterization difficult or impossible via most of the conventional characterization methodologies (e.g. chromatography such as SEC, electrophoresis, MALDI). TA could therefore provide easy means to characterize biohybrids as very recently shown for the first time in the Laboratory of Synthetic Biomaterials.[56]

Aim of this study was to analyse and further evaluate the applicability of TGA and (in a lesser extend) DSC on protein-polymer conjugate characterization. All samples were synthesized by members of the Laboratory of Synthetic Biomaterials, extensively dialyzed and freeze-dried in order to remove water. The samples were measured in the appropriate aluminium pans and mass was of the order of some. Importantly, all samples were subjected to an inert atmosphere (nitrogen) during their TA characterization. Below is a summary of the bioconjugates that are characterized in this Thesis (Table 1.2).

Table 1.2: Samples characterized in this Thesis.

Samples
BSA-Br
BSA-PS
BSA-PPgA
BSA-PPgA modified *via* CUAAC click chemistry
BSA-PMMA
PS-PMMA

Chapter 3: Experimental section

3.1 DSC measurements

The DSC experiments, presented in this thesis, follow the same procedure in all cases with the melting, glass transition and crystallization temperature to be the only differences during the experiments. In order to characterise a sample in a DSC analyser the sample is weighted. After that, the sample is enclosed in a pan and sealed with a press. Then, the pan is carefully placed in the DSC sample holder, while an identical reference pan is placed empty next to sample pan. Once the machine is closed, the DSC thermogram is recorded. This process is performed at a heating rate of 10℃/min. Inside the furnace an inert gas (usually nitrogen) is needed to flow for the proper performance of the experiment. When the experiment process is completed, the sample pan has to be removed from the furnace. From the above procedure, one diagram that provides information about the nature of the sample is obtained.

3.1.2 DSC experiment of BSA-PS

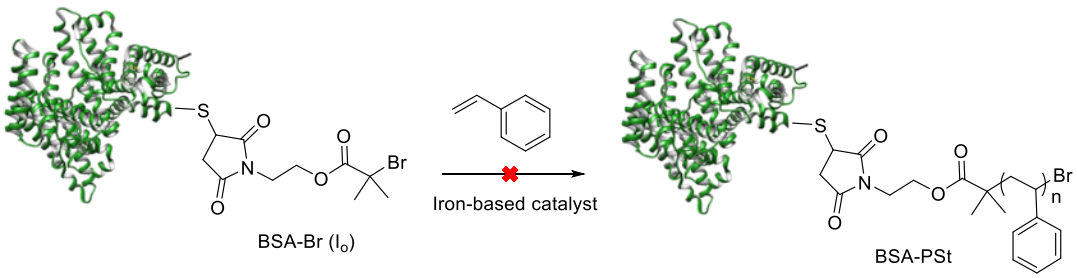


Figure 11: Attempt to synthesize BSA-PSt.

Four peaks were observed in the thermogram of the reaction product sample (Figure 12). As we saw to the TGA analysis before, BSA-PSt was not formed under the tested reaction conditions. Since the DSC and TGA analysis are related, we are

leading to the result that the curves that appear in the DSC thermogram refer to the stages our protein (BSA) passes through. First, an endothermic peak at 65.34°C (glass transition temperature) is attributed to the thermal denaturation of BSA-Br. Second another endothermic peak at 299.35°C was found and refers to the melting point of BSA-Br. Finally, the odd thing here, is that two exothermic peaks (as crystallization points) appear, one of which belongs to BSA-Br (246.43°C), while the other appears to belong to PS (174.86°C).[58] The truth is that if the experiment had been done at a wider temperature range, we might have had more conclusions for this synthesis.

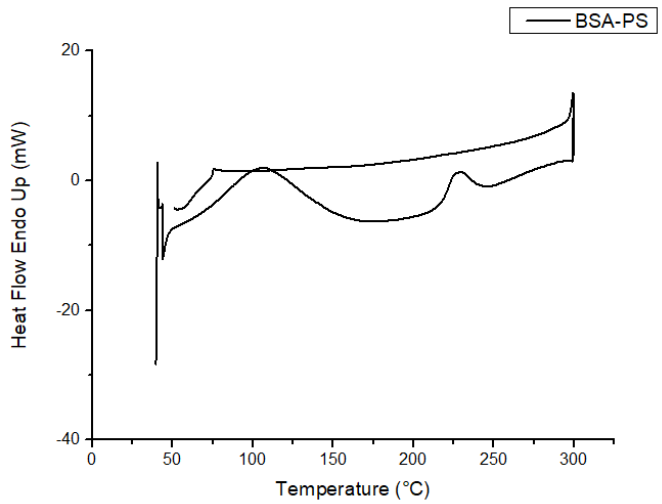


Figure 12: The DSC thermogram of BSA-PS.

3.1.3 DSC experiment of BSA-PS

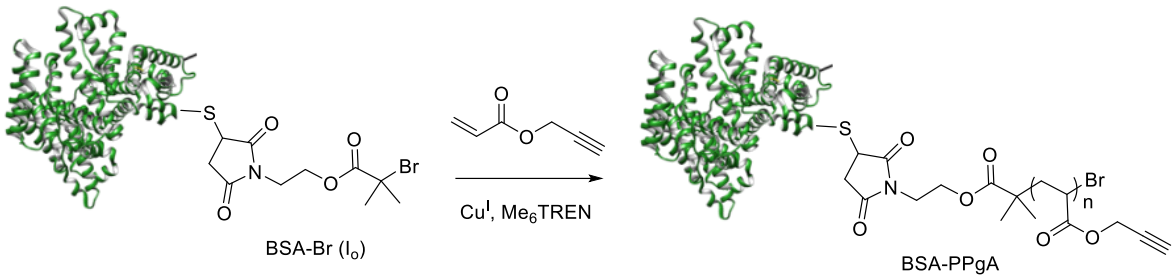


Figure 13: Synthesis of BSA-PPgA.

Four peaks were observed in the thermogram of the reaction product sample (Figure 14). As we saw to the TGA analysis before, TGA analysis of this sample indicates the successful synthesis of BSA-PPgA. Since the DSC and TGA analysis are related, the curves that appear in the DSC thermogram can lead to some conclusions. Firstly, an endothermic peak at 62.22°C is attributed to the thermal denaturation of BSA-Br. Secondly, another endothermic peak at 299.31°C was found and refers to the melting point of BSA-Br. Finally, one exothermic peak (the crystallization point) appears which belongs to PPgA (218.66°C).

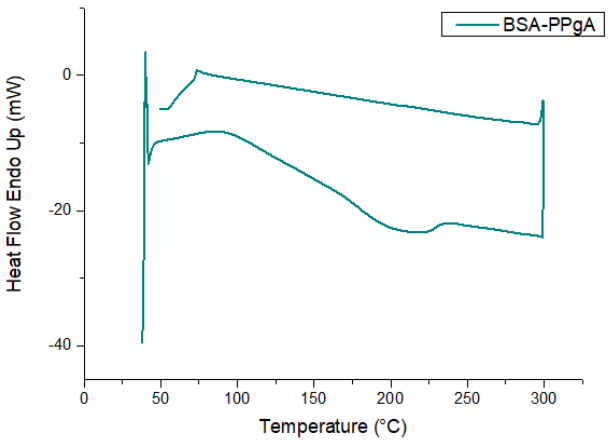


Figure 14: The DSC thermogram of BSA-PPgA.

In order to end up with some conclusions of the previous DSC experiments, we studied the DSC thermogram of the combination of BSA-PS and BSA-PPgA (Figure 15). As we saw at this thermogram, glass transition and melting temperatures are equal and only the crystallization temperatures of the biomolecules are different.

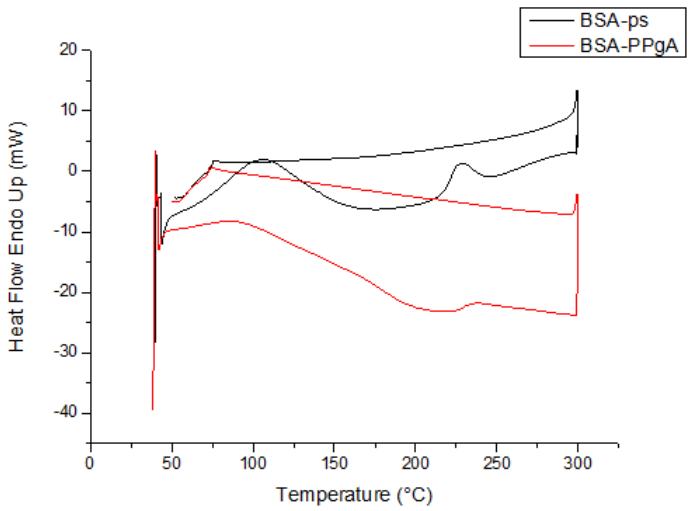


Figure 15: DSC thermograms of BSA-PSt and BSA-PPgA.

3.2 TGA measurements

The TGA experiments that are described in this Thesis have all been acquired using the same procedure with small variations at the initial and final temperature. In detail, two empty aluminium pans (the reference pan and the sample pan) were added to the furnace, the scale was reset to zero and the sample was placed on the sample pan. The TGA device was programmed with an initial temperature (T_i) and a final temperature (T_f) with a heating rate of 10°C/min. An inert gas, nitrogen was used in all measurements. The experiment process was complete within approximately 50 minutes and the sample pan was removed out of the furnace. Two diagrams providing information about the mass loss of the sample were obtained: the standard TGA thermogram and the derivative thermogram.

3.2.1 TGA experiment of BSA-Br

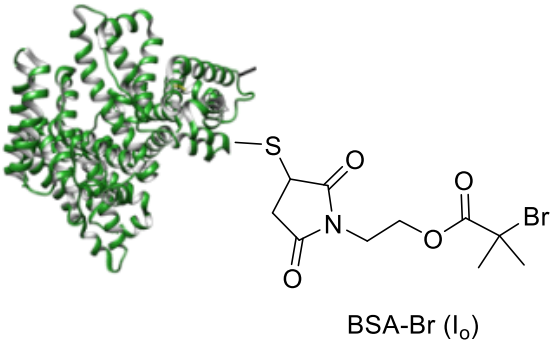


Figure 16: The chemical structure of BSA-Br.

In the case of BSA-Br, two weight loss stages were observed in the TGA thermogram. BSA-Br lost 10% of its weight during heating between 70.84 and 216.51 °C, which was attributed to the evaporation of the moisture and the denaturation of BSA (Figure 17).[59] In the second stage (216.51 to 447.15 °C), a significant weight loss of 60% could be attributed to the decomposition of BSA-Br.

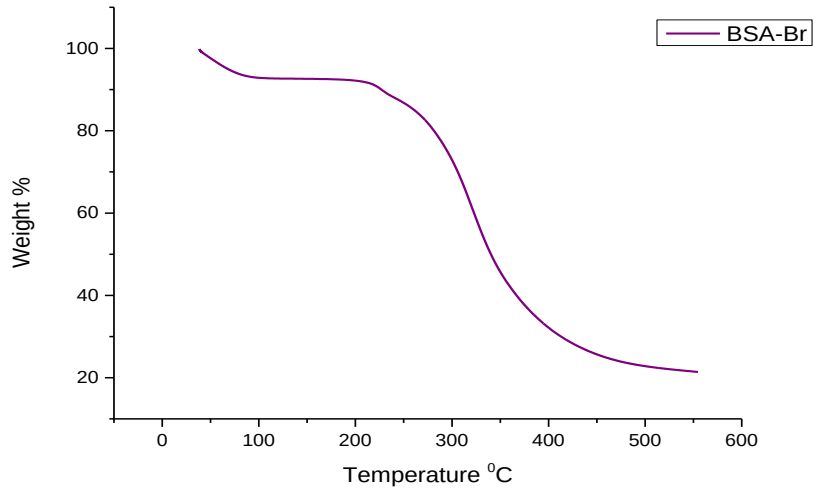


Figure 17: The TGA thermogram of BSA-Br.

Three curves were observed in derivative thermogram of BSA-Br (Figure 18). In accordance with the TGA thermogram, the first curve is attributed to the

evaporation of volatile components and moisture (<120^{o}C), while the second and the third curve (220-315^{o}C and 322^{o}C respectively) are attributed to the mass loss from BSA-Br degradation. The second curve could possibly be ascribed to the decomposition of the amide bonds, while the third curve to the decomposition of the aliphatic motifs of BSA-Br.[48] To conclude, the decomposition maximum rate occurs at T_d=322.32^{o}C.

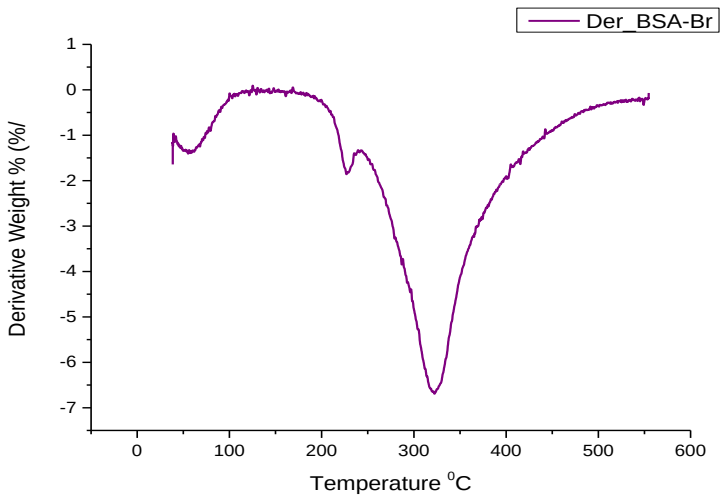


Figure 18: The derivative thermogram of BSA-Br.

3.2.2 TGA experiment of Polystyrene with MW: 10500

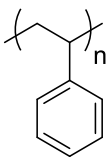


Figure 19: Chemical structure of Polystyrene.

Two weight loss stages were observed in TGA thermogram of Polystyrene (PSt). Polystyrene sample lost 4.9% of its weight when heated at 150 to 200 °C. The weight loss was attributed to the evaporation of volatile components and moisture.[60] In the second stage (400-440 °C), a significant weight loss of 95.1% could be attributed to the decomposition of PSt.

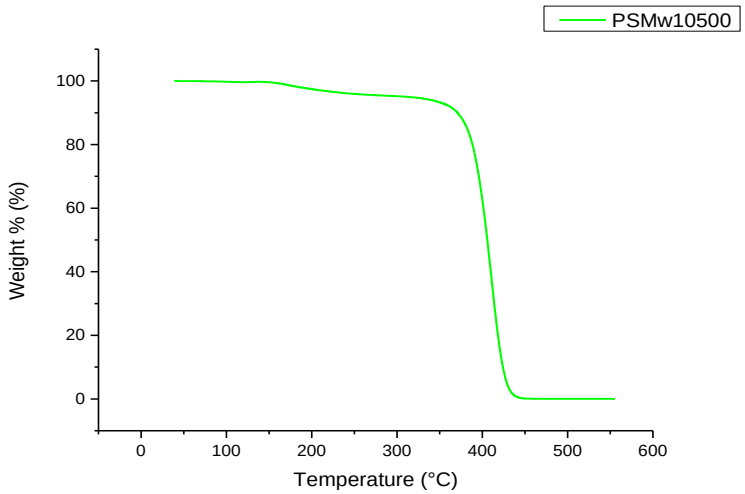


Figure 20: The TGA standard thermogram of PSt.

In accordance to the TGA thermogram, two curves were observed in derivative thermogram of PSt (Figure 21). The first curve represents very light volatiles components removal and moisture (<200^{o}C). Second curve represents the decomposition of PSt.[48]

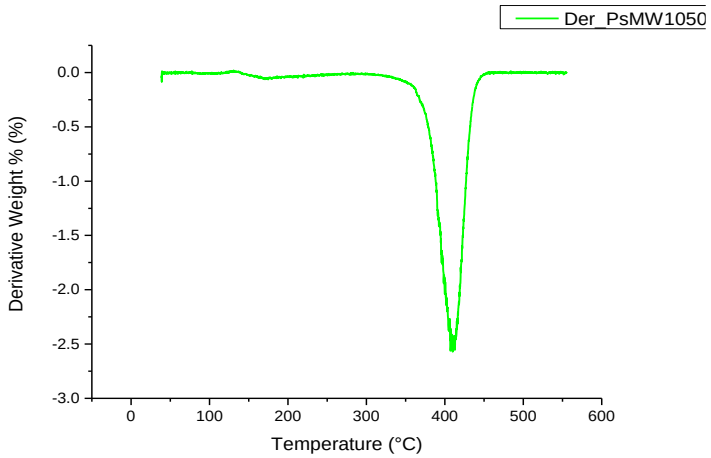


Figure 21: The DTG thermogram of PSt.

3.2.3 TGA experiment of BSA-PSt

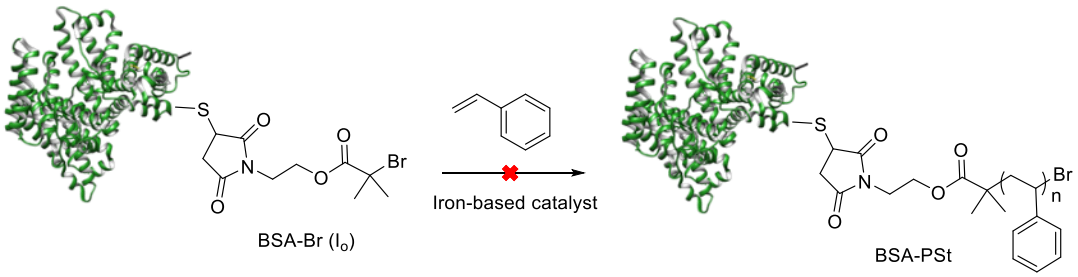


Figure 22: Attempt to synthesize BSA-PSt.

BSA-Pst was tried to be synthesized by grafting polystyrene from BSA-Br in the presence of an iron-based catalyst by the group of Prof. Velonia (Figure 22). The sample of the reaction product was characterized by TGA analysis. During the analysis of this sample, two weight loss stages were observed in the TGA thermogram. The reaction product lost 10% of its weight when heated at temperatures between 80 and 100 °C, which was attributed to the loss of the moisture and volatiles of the sample (Figure 23),[60] and a significant weight loss of 60% was observed when the sample heated at 200 to 300 °C, which could be attributed to the decomposition of functional amino groups within the side chains of BSA-Br. No weight loss was observed when the sample was heated at 400-440 °C,

indicating the absence of polystyrene in the sample. As a result, BSA-PSt was not formed under the tested reaction conditions.

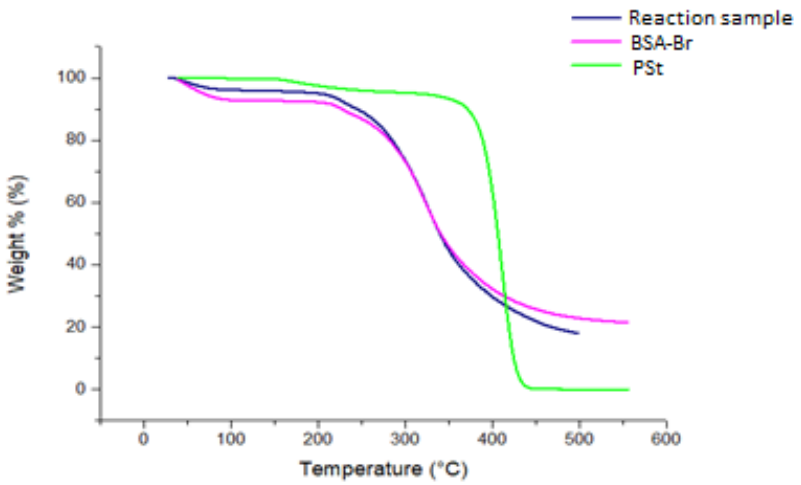


Figure 23: The TGA standard thermogram of the reaction sample, PSt and BSA-Br.

In agreement with the TGA thermogram, the derivative thermogram of the sample showed the same curves with the derivative thermogram of BSA-Br and the curve that is attributed to the decomposition of PSt was absent (Figure 24).

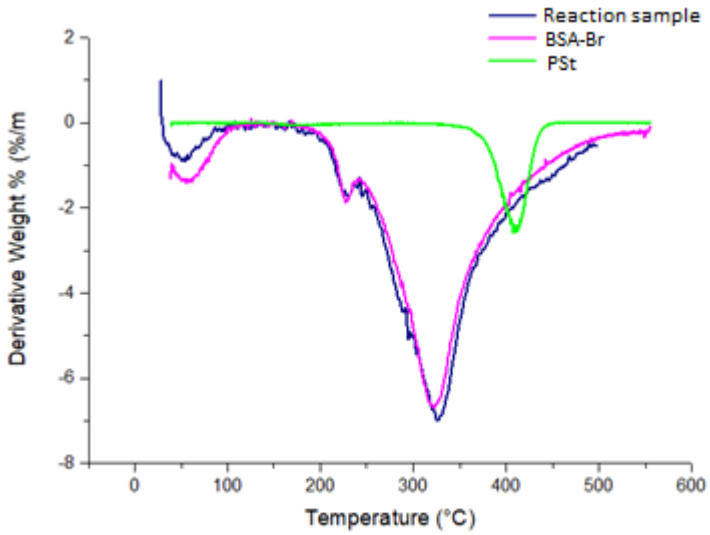


Figure 24: The DTG thermogram of the reaction sample, PSt and BSA-Br.

3.2.4 TGA experiment of BSA-PPgA

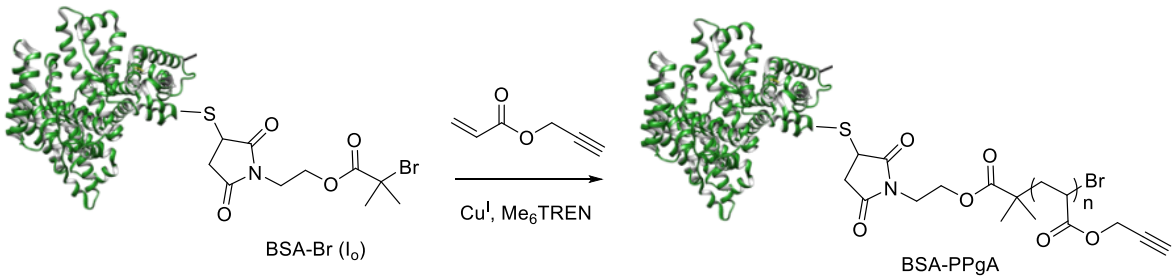


Figure 25: The synthesis of BSA-PPgA.

BSA-PPgA was synthesized by grafting poly(propargyl acrylate) from BSA-Br in the presence of an copper(I)-based catalyst by the group of Prof. Velonia (Figure 25). Three weight loss stages were observed in TGA thermogram of the reaction product sample. The product sample lost 1.7% of its weight when heated when heated at temperatures between 80 and 100 °C, which was attributed to the loss of the moisture and volatiles of the sample (Figure 26), a significant weight loss of 70% was observed when the sample heated at 200 to 300 °C, which could be attributed to the decomposition of functional amino groups within the side chains of BSA-Br. And a third weight loss was observed when the sample was heated at 350-400 ^oC, a curve that is not observed in the thermal analysis of the BSA-Br sample and can be attributed to the decomposition of primary esters of poly(propargyl acrylate).

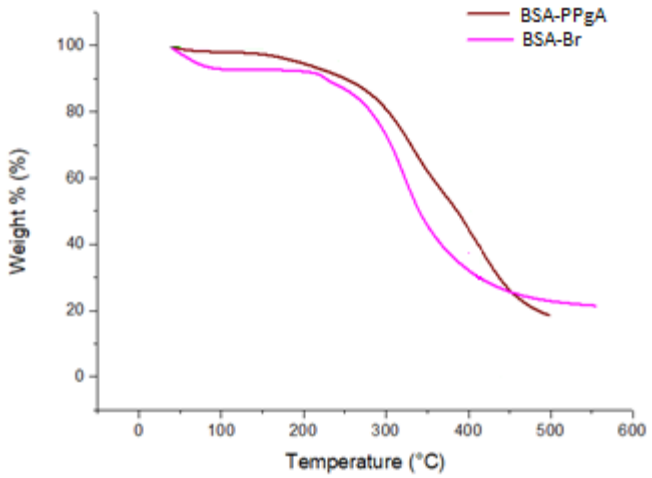


Figure 26: The TGA thermogram of BSA-PPgA and BSA-Br.

Three curves were also observed in the derivative thermogram of the above sample (Figure 27). The first curve represents very light volatiles components removal and moisture (<120^{o}C), second curve represents the decomposition of BSA (334.22^{o}C). Finally, third curve represents the decomposition of primary esters of poly(propargyl acrylate) (410.25^{o}C). As a result, TGA analysis of this sample indicates the successful synthesis BSA-PPgA.

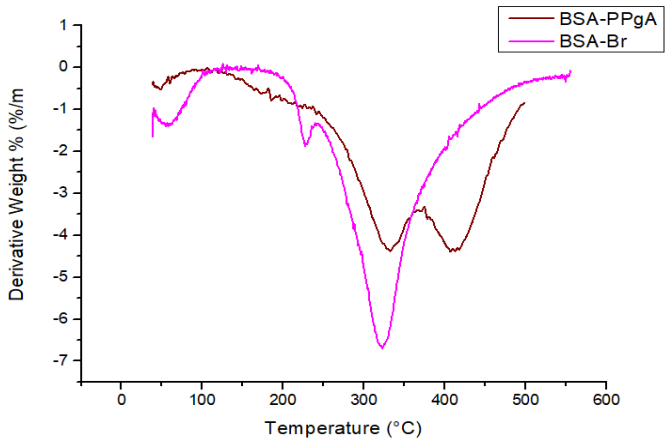


Figure 27: The DTG thermogram of BSA-PPgA and BSA-Br.

3.2.5 TGA experiment of modified BSA-PPgA *via* CUAAC click chemistry.

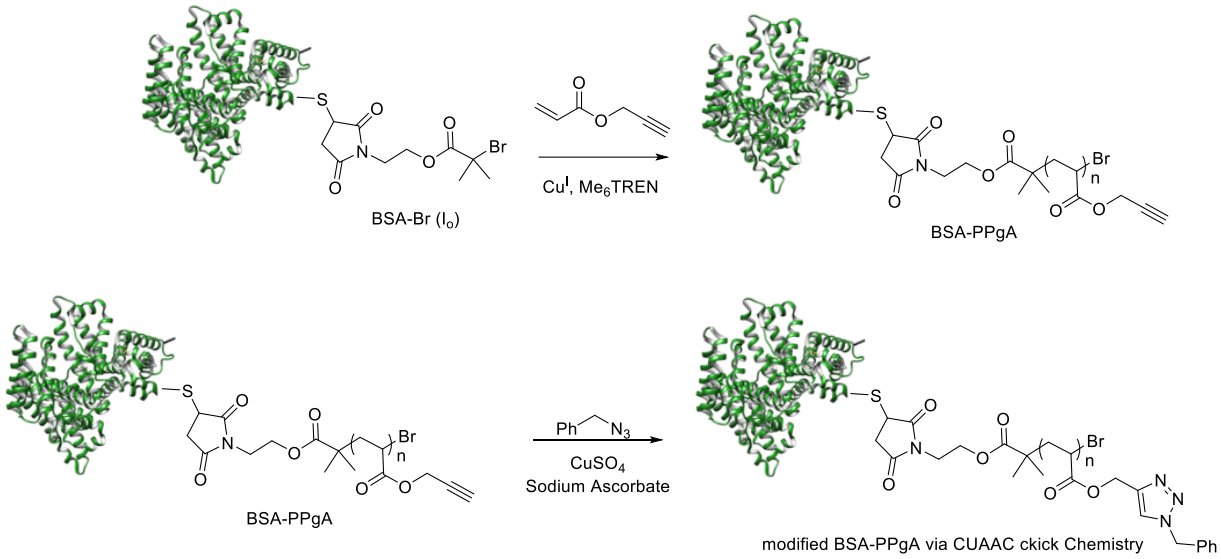


Figure 28: Two-pot synthesis of modified BSA-PPgA.

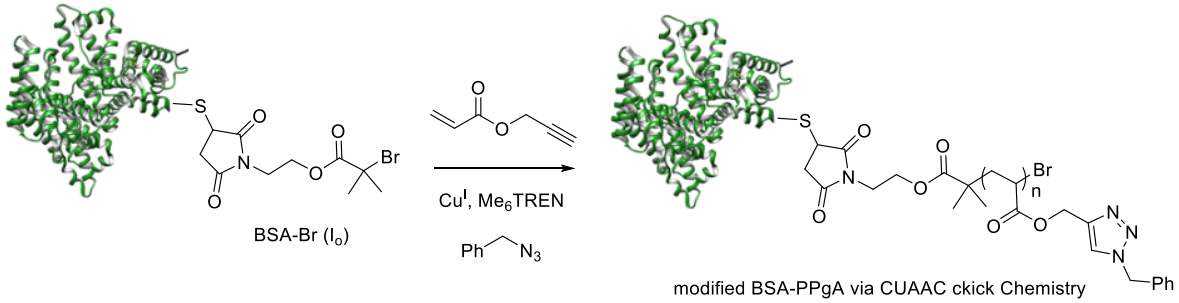


Figure 29: One-pot synthesis of modified BSA-PPgA.

BSA-PPgA was further functionalized *via* CUAAC click chemistry by both two- and one-pot procedures by the group of Prof. Velonia (Figures 28 and 29 respectively). Samples of the reaction products of both procedures were analysed by TGA thermal analysis. No significant differences were observed in TGA thermograms in both cases compared to TGA thermogram of BSA-Br (Figure 30). More specifically, a first weight loss of 10% was observed when the samples were

heated between 80 and 100 °C, which was attributed to the loss of the moisture and volatiles of the sample and a second weight loss of 70% was observed when the samples were heated at 200 to 300 °C, which could be attributed to the decomposition of BSA-Br. As a result, we are not able to get accurate results for the formation of the desired products from TGA diagrams.

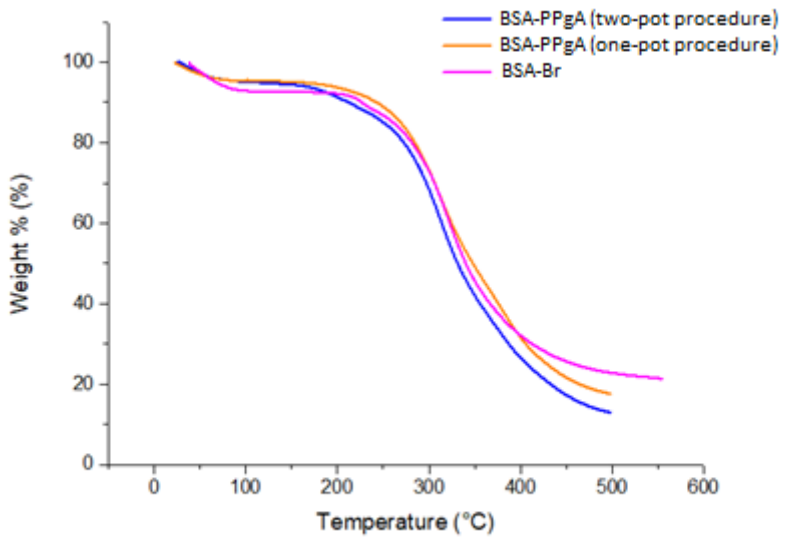


Figure 30: The TGA thermograms of modified BSA-PPgA via CUAAC click chemistry by both two- and one-pot procedures and BSA-Br.

Interestingly, in derivative thermograms of the above samples a new curve was observed (Figure 31). The first curve in derivative thermograms of both samples represents the volatile components and moisture removal (<120^{o}C), while the second one represents the degradation of the BSA (300-320^{o}C). Finally, a third curve that was absent in the derivative thermograms of BSA-PPgA can be attributed to the decomposition of the 1,2,3-triazole ring.

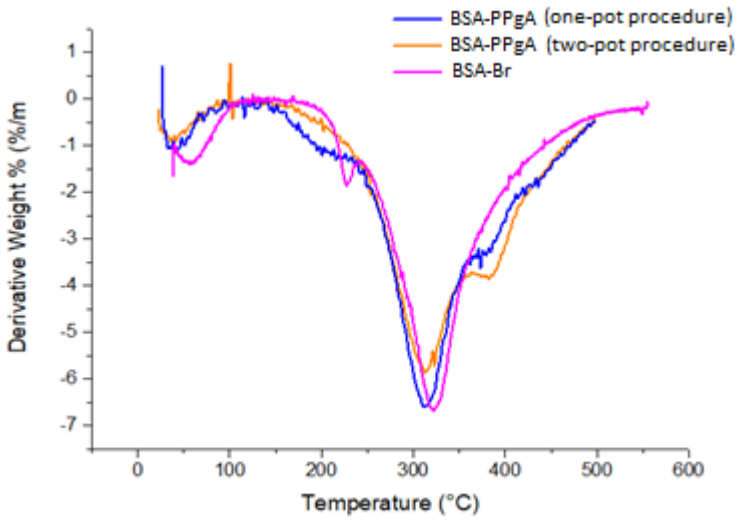


Figure 31: The TGA derivative thermograms of modified BSA-PPgA via CUAAC click chemistry by both two- and one-pot procedures and BSA-Br.

3.2.6 TGA experiments of BSA-Poly(methyl methacrylate) (BSA-PMMA).

The synthesis of BSA-PMMA was performed by Alexis Theodorou (Post-doctoral researcher of Prof. Velonia Lab). In this thesis, the characterization of BSA-PMMA by thermal analysis techniques will be described.

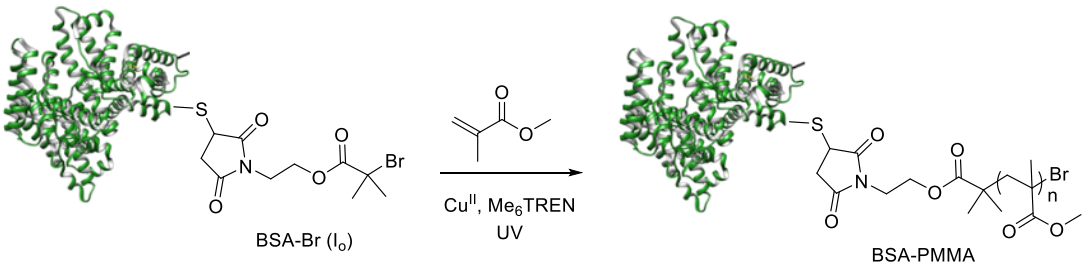


Figure 32: Organic-chemical reaction of BSA-PMMA.

Two weight loss stages were observed in the TGA thermogram of the BSA-PMMA sample (Figure 33). BSA-PMMA sample lost 20% of its weight when heated

at 200 to 300 °C, a loss that can be attributed to the BSA decomposition. In the second weight loss stage (390 to 450 °C), a significant weight loss of 68% could be attributed to the decomposition of PMMA.

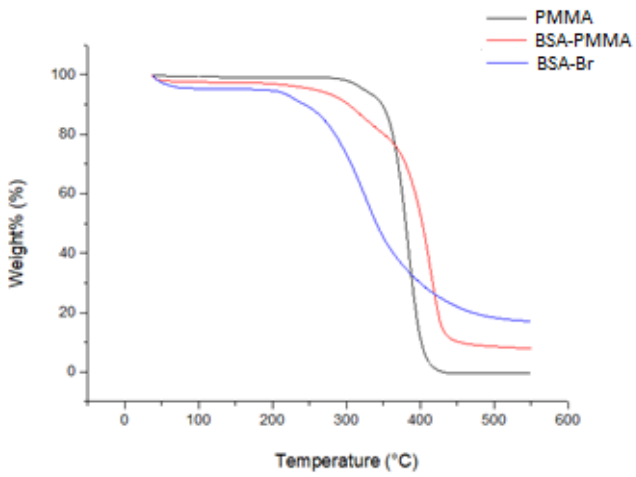


Figure 33: The TGA thermograms of BSA-PMMA, PMMA and BSA-Br.

In agreement with TGA thermograms, two curves were observed in derivative thermogram of BSA-PMMA sample (Figure 34). The first curve of BSA-PMMA represents the decomposition of BSA that exists in quite small amount in the sample, while the second curve represents the decomposition of PMMA.[61]

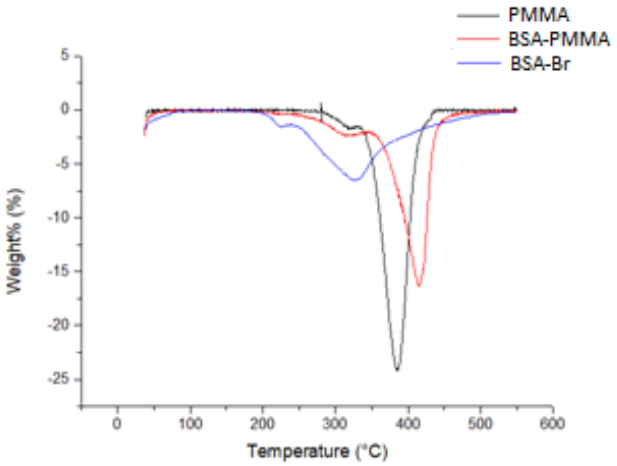


Figure 34: The TGA derivative thermogram of BSA-PMMA, PMMA and BSA-Br.

A commercially available PS-PMMA sample was also characterized by TGA analysis and compared to PMMA and PS.

In the TGA thermogram of PS-PMMA sample, a significant weight loss of 96.4% was observed in TGA thermogram at 400 ^{o}C, similar to the weight loss during the PMMA decomposition (Figure 358). This weight loss could indicate that the PS-PMMA sample is mainly composed of PMMA and in a lesser extent of PS.

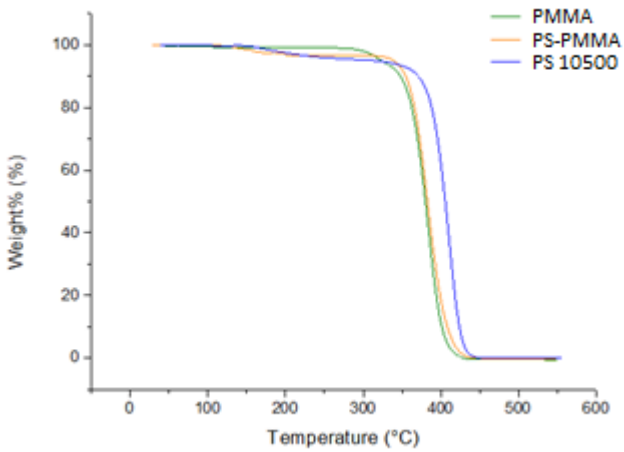


Figure 35: The TGA thermograms of PS-PMMA, PMMA and PS10500.

In accordance with TGA thermogram, the derivative thermogram of the PS-PMMA sample shows a large curve at 380^{o}C. As a result, the temperature of maximum rate decomposition is similar to the temperature of maximum rate decomposition of pure PMMA.

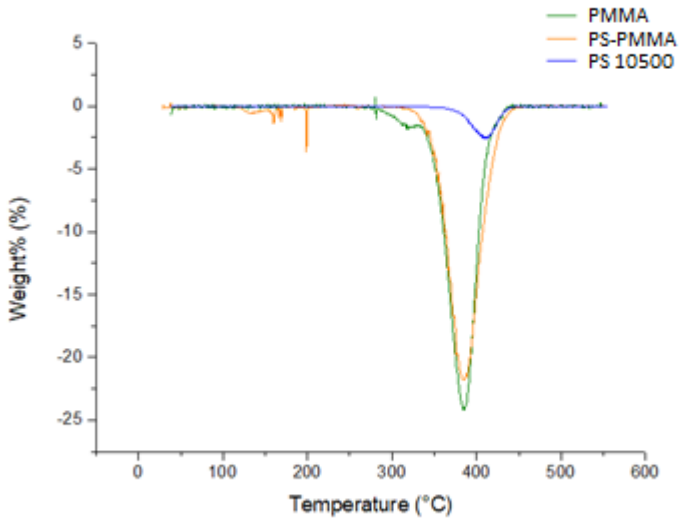


Figure 36: The TGA derivative thermogram of PS-PMMA, PMMA and PS10500.

Table 1.3 presents information about temperatures, which attributed at the 10% of the weight loss of each sample and the decomposition peaks of maximum rate, at TGA and DTG thermograms, respectively. From bibliography it is known that the higher temperature of 10% weight loss of a sample, the longer it takes for biopolymer to decompose.[62] This can be seen in our samples which gives us another argument that our experiments were done correctly, except for the sample PS-PMMA, because is not a biopolymer.

Table 1.3: Results of previous experiments.

Samples	$T_{10\%}$	Peaks of decomposition maximum rate.
BSA-PPgA(one pot procedure)	207.6 °C	310.95°C
BSA-Br	226.07°C	321.26°C
BSA-PPgA(two pot procedure)	240.62°C	312.6°C
BSA-PS	244.41°C	326.86°C
BSA-PPgA	247.25°C	412.86°C
BSA-PMMA	303.46°C	413.85°C
PS-PMMA	354.57°C	384.83°C

Chapter 4: Conclusions

The aim of the thesis was a bibliographical review of two thermal analysis processes (DSC and TGA). These processes/techniques, being two of the primary tools for thermodynamic analysis, were used for the characterization of protein-polymer conjugates, which were synthesized in Laboratory of Synthetic Biomaterials by the group of Prof. Velonia.

Due to the fact that this thesis is mainly bibliographic, not enough samples were characterized but even with them we managed to draw some conclusions about our samples. More specifically, the thermal analysis of our samples via Thermogravimetric Analysis (TGA) and Differential Scanning Calorimetry (DSC), provided us with thermograms which help us understand better these samples. In particular, the successful synthesis of the desired biomolecules was investigated and in some cases was confirmed. The ability of these thermal analysis techniques to determine the physical properties of different compounds including biomolecules was also verified.

Unfortunately, COVID affected us so that we could not examine enough samples, to arrive at more specific results of the synthesis of our samples.

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