ON SOME PRACTICAL ASPECTS OF CLICK THIOL-ENE REACTIONS FOR POLYMER MODIFICATION AND CONJUGATION WITH BIOMOLECULES

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ABSTRACT: Thiol-ene reactions are among the most popular click reactions, being particularly appealing for functionalization of polymer materials and conjugation with proteins. However, although the formal definition of "click chemistry" can be very strict, one can easily arise some controversial issues related to the application of thiol-ene reactions in actual process conditions. In order to illustrate some of these issues, thiol-ene reactions were performed at conditions that simulate the conjugation of proteins with unsaturated polymers. It is shown that the presence of a heterogeneous reacting system can exert enormous impact on the performance of thiol-ene reactions, which has been largely overlooked in the literature. It is also shown that the performance of heterogeneous click reactions depends on the many constituents of the reacting system so that the design of click reactions requires detailed analysis of the desired reaction system and may not necessarily constitute a mechanistic route for immediate bioconjugation applications.

KEYWORDS: Click Chemistry, Thiol-Ene, Polymer, Bioconjugation, FTIR.

INTRODUCTION

Click chemistry reactions have been studied extensively for 20 years, but were formally defined for the first time by KOLB and coworkers in 2001 [1]. These authors established that click reactions must necessarily be “modular, wide in scope, give very high yields, generate only inoffensive byproducts that can be removed by nonchromatographic methods, and be stereospecific (but not necessarily enantioselective). The required process characteristics include simple reaction conditions (ideally, the process should be insensitive to oxygen and water), readily available starting materials and reagents, the use of no solvent or a solvent that is benign (such as water) or easily removed, and simple product isolation.”[1].

Click chemistry reactions have been used since then to produce dendrimers [2-4], to modify polymers [5-7] and to conjugate biomolecules to different substrates [8-12]. In the particular case of polymer applications, the use of click chemistry reactions to modify the surface of polymer particles and to conjugate proteins with polymer chains is appealing, given the expected high yields and specificity of the chemical reactions.

The most commonly studied click chemistry reactions are: (i) Huisgen reactions; (ii) copper-free azide-alkyne cicloadditions; (iii) Diels-Alder reactions, (iv) thiol-ene and thiol-yne reactions; (v) condensation of aldehydes and ketones with heteroatom-bound amines; and (vi) Staudinger ligation of azides and phosphines [13]. Each distinct click reaction presents characteristic advantages and disadvantages that are briefly discussed below in the context of polymer modification and conjugation with proteins.
CuAAC reaction, also known as the Huisgen reaction, is undoubtedly the most studied click reaction. The use of a copper catalyst to perform the reaction constitutes simultaneously important advantage and disadvantage of this click reaction [12]. For example, the use of a copper catalyst makes the reaction $10^3$ times faster than the respective copper-free azide-alkyne cycloaddition. Besides, the use of the copper catalyst can enhance both reaction yields and product selectivity by orders of magnitude, when compared to the uncatalyzed reaction [14,15]. Copper, however, is associated to many diseases, including hepatitis, Alzheimer’s and other neurological diseases and cannot be removed from the product [16]. As a consequence, the use of the resulting products in pharmaceutical and biological applications is not advisable. Attempts have been made to develop and use heterogeneous copper catalysts in Huisgen reactions, but reports indicate that these catalysts seem to slow down the reaction significantly [17]. For this reason, currently the CuAAC reaction does not constitute a viable click reaction option for development of biomedical applications, including conjugation of polymer materials to proteins.

Copper-free azide-alkyne cycloadditions are more suitable for biomedical applications, due to the absence of copper. However, long reaction times and high temperatures are usually needed [15], which could constitute a major problem for biomolecule conjugations.

Regarding the Diels Alder reactions, one of its main disadvantages in bioconjugation applications is the fact that it may be necessary to modify the structure of the biomolecule [18]. Although in some cases the modification seems trivial from the chemical point of view, proteins, peptides and carbohydrates present complex three-dimensional structures that must be preserved, if preservation of the biological activity is also sought. In these cases, small changes of the chemical and geometrical structure of these molecules can result in an irreversible loss of activity [19]. As a consequence, Diels Alder reactions are used mainly for production of polymer materials with molecular structures that can be difficult to produce otherwise [20].

Condensation of aldehydes and ketones with heteroatom-bound amines was the first click reaction described as biorthogonal, which means that the reaction can occur inside a living system without interfering with native biochemical processes [21]. However, despite aldehyde and ketone functional groups are not present on the cell surface, they appear as intracellular metabolites. Because of that, the use of this type of reaction for labeling of biomolecules within living systems is limited [13,21]. Moreover, ketones and aldehydes react with amine nucleophiles that are enhanced by the π-effect, but also react with thiols and alcohols. Therefore, for bioconjugation applications, even though the reactions with thiols and alcohols are usually thermodynamically unfavorable in water, there is always a risk that residual aldehyde or ketone groups present in the conjugate system might interact with the living system [21,13]. Accordingly, condensation of aldehydes and ketones with heteroatom-bound amines does not seem to be truly biorthogonal.

As described in the previous case, Staudinger reactions are also biorthogonal, since none of its reactants or products interact with the biological environment [22]. However, handling of organic azides should be made with extreme caution, because most of them are explosive [13]. As a consequence, based on the original definition proposed by KOLB and coworkers, it is debatable whether the term "click" can be used to qualify this class of reactions.

Thiol-ene and thiol-yne reactions comprise addition reactions between a molecule containing a thiol group and alkenes or alkynes, respectively [23]. Some authors report that thiol-yne
reactions are about three times slower than thiol-ene reactions, particularly because thiol-yne reactions occur in two steps instead of one [24].

In the present manuscript, click thiol-ene reactions are discussed with more detail because they are faster than thiol-yne reactions and because many biomolecules contain cysteine residues [25], making biomolecule modification unnecessary. This can constitute a major advantage of this particular click route for bioconjugation. Additionally, thiol-ene reactions can occur through free radical initiation, be mediated by nucleophiles, acids and bases (known as thiol-Michael reactions) or simply require the presence of polar solvents, such as water. Moreover, the range of reactants that may be used is extensive and includes both activated and non-activated alkenes [24]. One must also consider that polymer chains produced with vinyl monomers normally present unsaturated pendant vinyl bonds in the molecule, encouraging the use of the thiol-ene route for bioconjugation.

The procedure most widely adopted to perform thiol-ene reactions makes use of thermal or photo radical initiators. The reaction pathway is similar to free radical polymerizations and includes initiation, propagation and termination steps [24]. The reaction mechanism is discussed in more detail further ahead, in a section specifically dedicated to this topic. It must be noted that the addition occurs with anti-Markovnikov orientation [24] and that molecules containing thiol groups act as chain transfer agents [26]. Reaction times are usually of the order of seconds even under mild temperature and pressure conditions. It is also worth mentioning that thiol-ene reactions are not usually affected by the presence of atmospheric oxygen, which can constitute a major practical advantage in many applications [27].

In the field of bioconjugation, the idea that a simple reaction with high efficiency and that does not require the use of toxic solvents can be exploited to immobilize biological assets onto the surface of polymer particles is extremely attractive and justifies the general interest in click chemistry. However, even though click reactions seem to be simple and effective, some authors have discussed intrinsic limitations of click reactions [28-30], while many aspects related to the practical implementation of click reactions in actual production environments have yet to be discussed.

One interesting aspect that has been consistently overlooked in the literature, for example, is the fact that most authors make use of toxic solvents in the proposed reaction schemes. This practice seems to contradict the original definition proposed by KOLB and coworkers in 2001 [1], although many researchers report the use of dichloromethane [31,32], chloroform [15,33], dimethylformamide [32,34], tetrahydrofuran [15,35,36], pyridine [15] and other toxic solvents [15,35,37] to perform click reactions. It is worth mentioning that solvent residuals must be completely removed from the final product when biomedical and pharmaceutical applications are sought, in order to avoid intoxication and meet quality-control requirements [38]. Product purification, however, should be performed with care (in order to preserve the biological activity of the molecule) and may involve a complex series of unit operations (distillation, filtration, centrifugation, among others) and, as a consequence, can be very expensive. For this reason, the use of toxic solvents should be avoided and it might be argued if reactions performed with toxic solvents should be really regarded as click chemistry procedures.

Another important point regards the fact that click chemistry reactions are normally performed in solution (homogeneous media), although bioconjugation reactions between polymer particles and biomolecules usually are performed in heterogeneous media. KOLB et al. (2001) observed that, when water is used as solvent, solubilization of reactants in the reaction medium...
may not be required [1]. As a matter of fact, KOLB et al. defended that efficient agitation should be sufficient to maintain solid materials homogeneously suspended in water and guarantee that reactions would take place efficiently [1,35]. DECAN et al. [39] studied the use of copper nanoparticles as heterogeneous catalyst for CuAAC click reactions and reported that only one catalytic event per nanoparticle took place at a given time and that product had a residence time of 3 s after the surface reaction. ZHAO et al. [40] studied a photochemical thiol-ene click modification of cellulose in its solid native state. In this reaction, solid cellulose was added to a water solution of 2,2-dimethoxy-2-phenylacetophenone and irradiated for one hour. Authors reported success in the proposed heterogeneous reaction.

In spite of that, the vast majority of the publications report that the analyzed reactions occur in homogeneous media [31-37]; however, it is not possible to assume that results obtained in solution would be similar in heterogeneous systems. As a matter of fact, some authors justify that their thiol-ene reactions were unsuccessful because reagents were incompatible with solvents, creating a heterogeneous reaction mixture [41]. As a consequence, it can be said that very little is known about click reactions performed in heterogeneous systems, as required by some bioconjugation reactions. It is important to emphasize that most polymer products are prepared in heterogeneous media and obtained as fine solid suspensions in water.

Regarding more specifically the click thiol-ene reactions, an important practical issue is related to the possible effect exerted by different free radical initiators on the course of the chemical transformations. The usual click thiol-ene mechanism does not require the detailed specification of the free radical initiator and this probably explains why AIBN (azobisisobutyronitrile) is used almost exclusively in all published procedures. As a matter of fact, few authors attempted to use other types of thermal initiators, such as potassium persulfate and benzoyl peroxide, in their studies, although the use of these initiators is much more usual in real production environments because of the reduced costs and safer handling procedures [42].

Moreover, click chemistry reactions already reported (and especially the ones related to polymer conjugation) are extremely complex and usually require multiple reaction and purification steps [35,43]. This makes the whole procedure very hard or nearly impossible to reproduce. In the present work, the main goal was to carry out heterogeneous thiol-ene reactions in a very simple way using readily available and non-toxic reagents (always as possible). To the best of our knowledge, no other published work has fulfilled these requirements.

Based on the discussion presented in the previous paragraphs, click thiol-ene reactions were performed between a divinyl monomer (divinylbenzene, 1,5-hexadiene or ethylene glycol dimethacrylate) and a molecule containing free thiol groups (L-cysteine, cysteine hydrochloride or 3-mercaptopropionic acid) in order to assess and illustrate some practical issues related to the use of click thiol-ene reactions for bioconjugation in heterogeneous media. The analyzed monomers are frequently used for reticulation of polymer particles and to provide unsaturated vinyl bonds for posterior polymer functionalization. The analyzed biomolecules frequently constitute proteins and are possible sources of thiol groups for bioconjugation. Reactions were conducted in aqueous suspensions, as usually desired at plant site, although toxic solvents were also used in some cases for benchmarking purposes. In order to illustrate the importance of the free radical initiator, reactions were performed using different thermal initiators (AIBN, benzoyl peroxide and potassium persulfate). Based on the obtained results, some important practical gaps in the click thiol-ene literature are detected and discussed,
providing suggestions for research related to the use of click chemistry for conjugation of polymer particles and biomolecules in the near future.

MATERIALS AND METHODS

Materials

Ethylene glycol dimethacrylate (EGDMA, with minimum purity of 98 wt%), 1,5-hexadiene (HD, with minimum purity of 97 wt% and containing up to 3 wt% of other diolefins), 3-mercaptopropionic acid (MPA, with minimum purity of 99 wt%), styrene (minimum purity of 99 wt%), dichloromethane (DCM, with minimum purity of 99.5 wt%), L-cysteine (Cys, with minimum purity of 97 wt%) and cysteine hydrochloride (Cys-Cl, with minimum purity of 98 wt%) were purchased from Sigma-Aldrich (Rio de Janeiro, Brazil). Benzoyl peroxide (BPO), sodium borohydride and N,N-dimethylformamide (DMF) were obtained from VETEC (Rio de Janeiro, Brazil) with minimum purity of 99 wt%. Azobisisobutyronitrile (AIBN) was purchased from Akzo Nobel (Netherlands) with minimum purity of 99 wt%. Divinylbenzene (DVB) was obtained from Merck (Germany) with purity of 99 wt% (as a 65 wt% solution in ethyl-styrene). Potassium persulfate was purchased from Proquimios (Brazil) with minimum purity of 99 wt%. Tetrahydrofuran (THF) was obtained from Tedia (Brazil) and dimethyl sulfoxide (DMSO) from Nuclear (Brazil), both with minimum purity of 99 wt%. Deuterated chloroform was purchased from Cambridge Isotope Laboratories (USA) with minimum purity of 99 wt%. Unless stated otherwise, reagents were used as received.

Reactions

Thiol-ene Reaction with Styrene

2.36 g of AIBN were initially dissolved in 15 g of DMF. Then, 0.42 g of styrene and 6.09g of MPA were added to this solution. The final solution was transferred to a round bottom flask attached to a condenser and kept at 80 °C for 4 hours under constant magnetic stirring (600 rpm). The product was dried for 72 hours in a recirculating oven at room temperature and then washed with acetone.

Thiol-ene Reactions with L-Cysteine

10 g of distilled water, 1.21 g of L-cysteine and either HD (0.21 g), DVB (0.33 g) or EGDMA (0.50 g) were added into a round bottom flash attached to a condenser. After mixing and reaching the desired temperature, BPO (0.61 g), AIBN (0.41 g) or K₂S₂O₈ (0.68 g) was fed into the flask. The reaction mixture was kept under constant magnetic stirring (600 rpm) at 85 °C or at 70 °C for 1h30min. The condenser temperature was kept at 10 °C and products were dried in a recirculating oven at room temperature for 24h.

Thiol-ene Reactions with Cysteine Hydrochloride

30 g of distilled water, 1.57 g of cysteine hydrochloride and 0.25 g of EGDMA were added into a round bottom flash attached to a condenser. After mixing and reaching the desired temperature, AIBN (0.82 g) or K₂S₂O₈ (1.35 g) was fed into the flask. The reaction mixture was kept under constant magnetic stirring (600 rpm) at 70 °C for 4 h. The condenser temperature was kept at 10 °C and products were dried in a recirculating oven at room temperature. In some cases, cysteine hydrochloride was reduced with sodium borohydride prior
to the reaction. When this procedure was conducted, Cys-Cl, water and 1.13 g of sodium borohydride were kept under constant magnetic stirring for 2 h at ambient temperature. After this step, the reaction proceeded as described previously.

**Polymerization Reactions**

Bulk polymerization reactions were conducted in test tubes. A solution containing 1 g of benzoyl peroxide and 25 g of styrene was prepared (when copolymerization was carried out, 0.5 wt% of styrene was replaced by EGDMA). Then, 2 g of the solution were added to each test tube and placed in an ethylene glycol bath heated at 85 ºC. Each tube was removed from the bath at specified reaction times (10 min, 30 min, 1 h, 1h30min, 2 h, 2h30min, 3 h, 3h30min, 4 h and 4h30min) and 10 drops of alcoholic hydroquinone solution (1% w/v) were added [44]. All products were dried first in a recirculating oven and then in a vacuum oven at room temperature.

**Thiol-ene Reactions with 3-Mercaptopropionic Acid**

0.2 g of previously prepared poly(styrene-co-divinylbenzene), containing pendant double bonds, 2.5 g of DMF, DCM or THF and 4 mg of MPA were added into a round bottom flash attached to a condenser. After mixing and reaching the desired temperature, AIBN (3 mg) was fed into the flask. The reaction mixture was kept under constant magnetic stirring (600 rpm) at 80 ºC for 4 hours.

**Characterization**

**Attenuated Total Reflectance Fourier Transform Infrared Spectroscopy (FTIR-ATR)**

FTIR analyses were conducted in a Thermo Nicolet 6700 (Thermo Fisher Scientific, USA) equipped with Smart Orbit™, an ATR accessory comprising a diamond crystal and a swivel pressure tower that ensures the application of consistent pressure on samples. Infrared analyses were performed in the mid-infrared region with spectral resolution of 4 cm⁻¹ at room temperature. Spectral data were reported as averages of 128 scans.

**Gel Permeation Chromatography (GPC)**

GPC analyses were performed at 40 ºC with help of a Viscotek (Malvern, United Kingdom) VE2001 chromatograph equipped with a Viscotek (Malvern, United Kingdom) VE3580 refractometric detector and a Viscotek (Malvern, United Kingdom) 2500 UV detector set at 255 nm and equipped with a deuterium lamp, a Shodex KF-G (Showa Denko K.K., Japan) pre-column, two Shodex KF-804 (Showa Denko K.K., Japan) columns and one Shodex KF-805 (Showa Denko K.K., Japan) column. The equipment was calibrated with polystyrene standards with molecular weights ranging from 376 to 1.0 x 10⁶ Da. Samples were prepared with concentration of 1 mg/mL in THF. Solutions were filtrated with a Teflon filter with pore size of 0.45 μm. The injection volume was equal to 200 μL and the operating flow rate was equal to 1 mL/min.

**Solubility Tests**

For solubility tests, 1 mL of solvent (water, THF, DMF, DMSO, hydrochloric acid, acetone or methanol) was mixed with 10 mg of product and left under mild agitation at ambient temperature for 48 hours. Then, samples were heated until 60 ºC. Solubility was checked visually through identification of suspended solid material.
THIOL-ENE REACTION MECHANISM

In this section, the thiol-ene reaction mechanism is presented in more detail in order to complement the introductory section and help explaining the results presented ahead. The thiol-ene reaction has been known since the early 1900s and its mechanism has been described in many excellent reviews [45-48]. The mechanism is summarized in Figure 1, where the initiation, propagation, chain transfer and termination steps are illustrated.

As one can see by comparing Figure 1 and Figure 2, the mechanism that describes the thiol-ene reaction is very similar to the mechanism normally used to represent a free-radical polymerization reaction. The main difference relies on the initiation step. In the polymerization mechanism, initiation consists in the decomposition of the initiator, generating free radicals that readily react with monomers. In the thiol-ene mechanism, the free radicals generated by the initiator decomposition react with the molecules that contain SH groups. Besides, it is important to highlight that molecules containing SH groups act as chain transfer agents.

In fact, the reaction of radical fragments with vinyl monomer molecules or thio-compounds constitutes a random event. Therefore, one cannot discard beforehand the occurrence of monomer initiation (or polymerization reactions) when thiol-ene reactions are pursued. In spite of that, this important side reaction has been usually neglected or omitted from mechanisms presented in publications concerning thiol-ene click reactions. CRAMER et al. studied the occurrence of polymerization reactions in click chemistry systems and reported that, when acrylate conversions reached 100%, thiol conversions were close to 50%, indicating the importance of this side reaction step [50]. This result confirms that the polymerization reaction occurred in a much bigger extent than the thiol-ene reaction. For this reason, the possible occurrence of polymerization reactions (as side reactions) will always be considered in the results presented in the following sections.
Figure 1. Schematic representation of the thiol-ene reaction mechanism.
Figure 2. Schematic representation of the free-radical polymerization reaction mechanism [49].

RESULTS AND DISCUSSION

Thiol-ene Reactions with Styrene

The first experiments performed in the present work intended to describe a benchmark system to be used as reference for all other proposed and analyzed reactions. As widely discussed in the click chemistry literature, the reactivity of the double bond molecules follows the following decreasing order of reactivity: norbornene > vinyl ether > vinyl ester > allyl ether > acrylate > N-substituted maleimide > methacrylate > styrene > conjugated dienes [45–48]. According to this scale, styrene can be successfully used in thiol-ene reactions and for this reason it was selected as a reactant for reaction A, as styrene constitutes a cheap and well-studied model polymerization system. After extensive review on thiol-ene reactions, it was observed that the equivalence thiol:ene ratios usually range from 2:1 to 10:1 in publications that reported high reaction yields and good selectivity [51,52]. Similarly, equivalence initiator:thiol ratios usually range from 0.1 to 1 [51,52] and the most commonly used thermal initiator is AIBN [35,37,51]. Finally, many groups reported successful results when MPA was used as the source of thiol groups [7,42,53]. Therefore, in reaction A, styrene and MPA were used in the equivalence thiol:ene proportion of 10:1, using AIBN as the initiator with the equivalence initiator:thiol ratio of 1:2.

Reaction A was conducted in solution, using DMF as solvent (as usual in the literature) and the final product was dried in a recirculating oven at room temperature and then washed with acetone before FTIR analysis. The FTIR analysis of the residual liquid product (after drying for 72 hours, some residual liquid still remained in the flask) was also performed. Both spectra are presented in
As one can see in Figure 3, a band placed at 699 cm\(^{-1}\) appears in both liquid and solid products of reaction A. This is a clear indication that the click chemistry occurred, since the C-S FTIR bands appear at 692 cm\(^{-1}\) [54]. The observed shift is common in FTIR analyses and can be related to interactions with other components. The residual liquid product of reaction A is a mixture of DMF, MPA and click chemistry product. It is important to observe that neither the solid product nor the liquid product present evidences of residual styrene double bonds, that should appear at 1630 cm\(^{-1}\), 910 cm\(^{-1}\) or 990 cm\(^{-1}\) [55]. Therefore it can be assumed that styrene molecules were efficiently converted into click products.

![FTIR spectra of liquid and solid products of reaction A and of MPA, DMF and styrene.](image)

In order to observe if polystyrene might have been produced as a side product, GPC analysis was performed with the solid product and RI and UV signals were detected. The GPC results for the solid product of reaction A are presented in Figure 4 and in Figure 5. As one can see in Figure 4, only one product was detected in the GPC analysis and the calculated number average molar mass was equal to 222 g/gmol. This is a clear indication that polymerization reactions did not take place; otherwise, the average molar mass would be much higher. Besides, the polydispersity index (PDI) was very small and equal to 1.08, indicating that nearly 100% of the molecules have the same size. This also indicates polystyrene was not formed, since in non-controlled polymerizations the PDI index is usually close to 2 or higher [49].

Figure 4 also indicates that the click product seems to be pure, since only one narrow peak was detected during the whole analysis. This is confirmed in Figure 5, which shows that the UV detector also detected a single peak that started to appear at the exact time when the RI peak was detected. Finally, the molar mass obtained by the GPC analysis was essentially equal to the sum of styrene and MPA molar masses (MPA= 106.14 g/gmol and Styrene = 104.15), suggesting once more that the reaction product was a click
product between styrene and MPA. Therefore, reaction A can be used as a successful benchmark for the other reactions performed in the present work, since it seems to constitute a successful thiol-ene system.

![Graph](image)

**Figure 4.** Molar mass distribution of the product obtained in reaction A

![Graph](image)

**Figure 5.** Responses of RI and UV GPC detectors for the solid product of reaction A.

In the following sections, several modifications are proposed in our benchmark reaction in order to adapt it to more realistic conditions concerning bioconjugation applications. In each section the modifications are presented and explained in detail.

**Thiol-ene Reactions with L-Cysteine at 85 °C**

In bioconjugation reactions, the thiol concentration is expected to be small, as thiol groups are usually provided by biomolecules (which are frequently extremely expensive) while the unsaturated double bonds are normally provided by the polymer matrix. As a consequence, thiol:ene ratios are expected to be much lower in real conjugation reactions involving polymer matrices and proteins, for instance. Also, it is not viable to use such high concentrations of toxic solvents as used in reaction A when biomedical and pharmaceutical applications are pursued. In fact, the best solution is always to use aqueous environments. Moreover, MPA decreases gamma-aminobutyric acid in the brain, thereby causing convulsions [56]. For this
reason, in this section MPA was replaced by L-cysteine, which is non-toxic and is present in the chemical structure of many biomolecules. Additionally, styrene is a monounsaturated monomer and cannot be used to insert pendant double bonds in a polymer matrix for further bioconjugation. In order to do so, multifunctional monomers must be used. Therefore, 1,5-hexadiene, divinylbenzene and ethylene glycol dimethacrylate were tested. Finally, all reactions described in this section were performed with 3 different initiators in order to compare the relative performances of the distinct initiation systems.

Based on the modifications proposed above, a first group of reactions was performed with different dienes, with equivalence thiol-ene ratio of 2:1 and using different thermal initiators with equivalence initiator:thiol ratio of 0.5, as shown in Table 1. Reactions were conducted in heterogeneous aqueous media at 85 °C. It is important to highlight that in this proposed group of reactions only K₂S₂O₈ is completely water soluble, while L-cysteine is poorly soluble in water. All other reagents are not water soluble, which means that in some cases the system is composed of three different phases (organic liquid phase, aqueous liquid phase and solid phase). Finally, L-cysteine is insoluble in HD, DVB and EGDMA. It is important to observe that the heterogeneous nature of the reacting system should be expected in most bioconjugation problems.

FTIR analyses of all obtained products and respective reagents were performed. Figure 6 shows the FTIR spectra of products obtained in runs 1, 2 and 3, performed with 1,5-hexadiene and different initiators. Spectra of HD and L-cysteine are also shown in Figure 1 for the sake of comparison. Figure 6 indicates that the chemical nature of the thermal initiators exerted an enormous influence on the reaction course, since the FTIR spectra of the obtained products were very different from each other. This point has been largely overlooked in the literature and clearly indicates that different initiators can exert distinct effects on the click thiol-ene reaction, making necessary the more detailed description of radical formation and radical interaction with the remaining reagents. It is also important to observe that none of the obtained solid products was soluble in the analyzed solvents, indicating the production of a complex mixture of products, as also suggested by the obtained FTIR spectra, which has also been overlooked in the literature. In other words, the expected high specificity of the click reactions is not attained in the present case, despite the usual reaction conditions and recipe. Because of the complexity of the obtained FTIR spectra, spectral analysis was focused mainly on the characteristic bands that can be related directly to the thiol-ene reactions.

The characteristic band that indicates the presence of C-S bonds is positioned at 692 cm⁻¹ [53]. Assuming that the click reaction occurred, one might expect the relative increase of this peak at the end of reaction. However, as indicated by the dotted lines in Figure 6, this increase could not be observed. It is also important to emphasize that the characteristic band related to S-S bonds is located at 538 cm⁻¹ [57], as also indicated by dotted lines in Figure 1. It is interesting to observe that the relative intensity of this band remained virtually unchanged after reaction, which apparently excludes the possible formation of cystine through reaction between two cysteine molecules [58]. Thus, one may possibly conclude that the click thiol-ene reaction between cysteine and 1,5-hexadiene, mediated by the analyzed thermal initiators, did not occur in heterogeneous aqueous media, as the characteristic C-S band was not detected. As important as that, the complexity of the FTIR spectra of final solid products and solubility tests indicated that other complex reactions took place, suggesting that the thiol-ene reaction may not present high specificity at the analyzed conditions. Figure 6 also indicates that 1,5-hexadiene double bonds were consumed during reactions 1, 2 and 3, since the peaks located at 910 and 990 cm⁻¹
and related to the double bonds of HD do not appear in the products. This consumption might be the result of 1,5-hexadiene polymerization, for example.

Table 1. Thiol-ene reactions with L-cysteine.

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Alkene (ene)</th>
<th>Initiator</th>
<th>Thiol</th>
<th>Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>HD</td>
<td>BPO</td>
<td>L-cysteine</td>
<td>85 °C</td>
</tr>
<tr>
<td>2</td>
<td>HD</td>
<td>AIBN</td>
<td>L-cysteine</td>
<td>85 °C</td>
</tr>
<tr>
<td>3</td>
<td>HD</td>
<td>K₂S₂O₈</td>
<td>L-cysteine</td>
<td>85 °C</td>
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<tr>
<td>4</td>
<td>DVB</td>
<td>BPO</td>
<td>L-cysteine</td>
<td>85 °C</td>
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<tr>
<td>5</td>
<td>DVB</td>
<td>AIBN</td>
<td>L-cysteine</td>
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<tr>
<td>6</td>
<td>DVB</td>
<td>K₂S₂O₈</td>
<td>L-cysteine</td>
<td>85 °C</td>
</tr>
<tr>
<td>7</td>
<td>EGDMA</td>
<td>BPO</td>
<td>L-cysteine</td>
<td>85 °C</td>
</tr>
<tr>
<td>8</td>
<td>EGDMA</td>
<td>AIBN</td>
<td>L-cysteine</td>
<td>85 °C</td>
</tr>
<tr>
<td>9</td>
<td>EGDMA</td>
<td>K₂S₂O₈</td>
<td>L-cysteine</td>
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<td>10</td>
<td>-</td>
<td>BPO</td>
<td>L-cysteine</td>
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<tr>
<td>11</td>
<td>-</td>
<td>AIBN</td>
<td>L-cysteine</td>
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<tr>
<td>12</td>
<td>-</td>
<td>K₂S₂O₈</td>
<td>L-cysteine</td>
<td>85 °C</td>
</tr>
</tbody>
</table>

It is interesting to observe that the FTIR spectrum of the solid product prepared with 1,5-hexadiene, L-cysteine and benzoyl peroxide in run 1 was more similar to the original FTIR spectrum of L-cysteine, when compared to the other two products. This may be related to the reaction temperature, as BPO decomposes more slowly than AIBN and potassium persulfate at 85 °C [60]. The possible effect of the rate of decomposition of thermal initiators on the course of click thiol-ene reactions has also been overlooked in the published material. The faster rate of radical generation provided by AIBN and persulfate may have led to higher concentrations of free radicals in the medium and possibly increased the importance of other undesired side reactions.
Figure 6. FTIR spectra of the final solid products of runs 1, 2 and 3 and of reagents L-cysteine and 1,5-hexadiene.

The FTIR spectra of solid products obtained through reactions of L-cysteine with DVB and EGDMA are shown respectively in Figure 7 and Figure 8. Once again it is possible to observe the distinct effects exerted by the different initiators on the reaction product and the fact that the relative intensity of the characteristic C-S band placed at 692 cm$^{-1}$ did not increase. As a consequence, one can say that the observed initiator effects and the low specificity of the thiol-ene reaction do not depend solely on the particular analyzed diene, which can be regarded as a very important conclusion. Similarly, the final FTIR spectra of solid powders obtained with BPO were more similar to the FTIR spectrum of L-cysteine than in the other cases, suggesting that the observed BPO effect does not depend on the analyzed diene either. Additionally, obtained solid products were not soluble in any of the analyzed solvents, as in the previous case. It is also important to emphasize that, as shown in Figure 7, the band associated to DVB pendant double bonds (985 cm$^{-1}$) [61] does not appear on the products of reactions 4, 5 and 6. This is a clear indication that the double bonds were consumed in side reactions that might include DVB polymerization, for example.

Figure 8 shows similar results for EGDMA, as the band related to the EGDMA double bonds (1641 cm$^{-1}$) [62] and indicated by a dashed line does not appear on the products of reactions 4, 5 and 6. This is once more a clear indication that the double bonds were consumed in side reactions that might include the EGDMA polymerization. Also, the presence of a band marked by a dashed line at 1720 cm$^{-1}$ [63], which can be associated to carbonyl groups, indicates the presence of EGDMA in the final product. This result seems to corroborate the idea that one of the side reactions that occur during the above described click chemistry attempts might be the polymerization of the dienes.
Figure 7. FTIR spectra of the final solid products of runs 4, 5 and 6 and of reagents L-cysteine and DVB.

Figure 8. FTIR spectra of the final solid products of runs 7, 8 and 9 and of reagents L-cysteine and EGDMA.

Figure 9 shows that the FTIR spectra of the solid products obtained with BPO were relatively similar, presenting lower sensitivity to the analyzed diene. The scenario is similar when the other initiators are taken into consideration, as shown in Figure 10 and Figure 11. Figures 4, 5 and 6 reinforce that the reactions performed with different initiators can follow different reaction mechanisms and suggest that the diene may not react with L-cysteine when the reagents are contained in distinct phases, as the dienes were suspended in the aqueous phase as small droplets.
Figure 12 shows FTIR spectra of solid products obtained in runs 10, 11 and 12, performed in absence of the dienes. Based on the FTIR data, it can be observed that the obtained products were different from the ones presented in Figures 1 to 6. This clearly shows that the dienes do take part in the reaction, but illustrates once more that the different initiators interact differently with L-cysteine, that the final solid material can be constituted by a mixture of different products, that the thiol-ene reaction can present low specificity and that the characteristic C-S bond may not be formed at all at the analyzed conditions. Additionally, obtained solid products were not soluble in any of the analyzed solvents.

Figure 9. FTIR spectra of the final solid products of runs 1, 4 and 7 and of reagents L-cysteine and BPO.

Figure 10. FTIR spectra of the final solid products of runs 2, 5 and 8 and of reagents L-cysteine and AIBN.
Based on the previous paragraphs, it sounds reasonable to say that thiol-ene reactions do not present the characteristic features of click reactions at the analyzed conditions. Besides, it seems clear that both initiator and diene affect the course of the reactions, which may lead to formation of a complex mixture of products, with low specificity towards formation of the desired C-S bond. These effects had never been discussed in the open literature and are very important for the proper design of heterogeneous bioconjugation systems.

**Figure 11.** FTIR spectra of the final solid products of runs 3, 6 and 9 and of reagents L-cysteine and K₂S₂O₈.

**Figure 12.** FTIR spectra of the final solid products of runs 10, 11 and 12 and of reagents L-cysteine, BPO, AIBN and K₂S₂O₈.
Thiol-ene Reactions with L-Cysteine at 70 ºC

In order to evaluate the effect of temperature (and of rate of free radical generation), experiments were performed with AIBN and K₂S₂O₈ at 70 ºC, as presented in Table 2. BPO was not used because the rate of thermal decomposition of BPO at 70 ºC is very low. DVB and EGDMA were used as monomers in these tests because these monomers are used more frequently as crosslinking agents in most commercial polymerization reactions. Once again it is important to highlight that in this second group of reactions performed at 70 ºC only K₂S₂O₈ and EGDMA are completely water soluble, while L-cysteine is poorly soluble in water. All other reagents are not soluble in water, which means that in some cases the system is composed of three different phases.

Table 2. Group of experiments performed at 70 ºC.

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Alkene (ene)</th>
<th>Initiator</th>
<th>Thiol</th>
<th>Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>13</td>
<td>DVB</td>
<td>AIBN</td>
<td>L-cysteine</td>
<td>70 ºC</td>
</tr>
<tr>
<td>14</td>
<td>EGDMA</td>
<td>AIBN</td>
<td>L-cysteine</td>
<td>70 ºC</td>
</tr>
<tr>
<td>15</td>
<td>-</td>
<td>AIBN</td>
<td>L-cysteine</td>
<td>70 ºC</td>
</tr>
<tr>
<td>16</td>
<td>DVB</td>
<td>K₂S₂O₈</td>
<td>L-cysteine</td>
<td>70 ºC</td>
</tr>
<tr>
<td>17</td>
<td>EGDMA</td>
<td>K₂S₂O₈</td>
<td>L-cysteine</td>
<td>70 ºC</td>
</tr>
<tr>
<td>18</td>
<td>-</td>
<td>K₂S₂O₈</td>
<td>L-cysteine</td>
<td>70 ºC</td>
</tr>
</tbody>
</table>

As one can see in Figure 13 and in Figure 14, formation of the characteristic C-S bond could not be detected in any run. Residual double bonds could not be detected either (band at 1641 cm⁻¹ for reactions 14 and 17 and 985 cm⁻¹ for reactions 13 and 16) [61,62], indicating that the dienes were consumed and formed byproducts. In reactions 14 and 17 the presence of the band at 1720 cm⁻¹ [62], which can be assigned to carbonyl groups, indicates the presence of EGDMA in the final product. One can also observe that the reaction temperature clearly affected the FTIR spectra of the final solid products, reinforcing the idea that the rate of free radical generation does affect the course of the thiol-ene reaction. Although this point has been largely overlooked in previous studies, it is not surprising, as the rate of radical generation affects the concentration of free radicals and the possible occurrence of undesired side reactions. Figure 15 illustrates the effect of temperature on FTIR spectra of the final solid powder.

One of the possible causes for absence of the characteristic thiol-ene reactions in the previously reported experiments could be the lack of free thiol in commercial L-cysteine. As reported in the literature, L-cysteine can react to form dimers, with formation of disulfide bonds [58]. As one can see in Figure 16, disulfide bonds can be detected through the characteristic S-S band placed at 534 cm⁻¹. Therefore, it may be important to reduce L-cysteine prior to use in click thiol-ene reactions, in order to eliminate possible disulfide bonds. This makes the commercial process less competitive and does not change the fact that a complex network of reactions seems to take place when the reagents are put in contact.
Another possible cause for absence of the characteristic thiol-ene reactions in the previously reported experiments could be the fact that reactions that were performed with AIBN (the most successfully used thermal initiator reported in the literature for thiol-ene reactions) were conducted in heterogeneous aqueous media, instead of homogeneous organic media, as reported by most authors. However, as discussed before, performing the thiol-ene reaction in homogeneous medium would constitute a major disadvantage for conjugation of polymer materials and biomolecules, as medical and pharmaceutical applications usually require
production of micro- and nanoparticles, which are produced in heterogeneous media during polymerization.

Figure 15. FTIR spectra of the final solid products of runs 11 and 15 and of reagent L-cysteine.

Figure 16. FTIR spectra of commercial L-cysteine.
Thiol-ene Reactions with Cysteine Hydrochloride

As L-cysteine is not very soluble in water, cysteine hydrochloride was used to perform the experiments presented in Table 3 in order to evaluate whether results could be different when molecules containing thiol groups were solubilized in the aqueous medium. In all these experiments, cysteine hydrochloride was first reduced with borohydride in order to prevent the existence of disulfide bonds and increase the amount of free thiol groups, as discussed previously. Cysteine hydrochloride, EGDMA and K$_2$S$_2$O$_8$ are water soluble. All other reagents are not soluble in water, which means that in reactions 19 and 20 the system was composed of two different phases, while in reactions 21 and 22 all reagents were water soluble, as in the benchmark click reaction performed with styrene, MPA and AIBN in DMF.

**Table 3. Reactions with Cys-Cl.**

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Alkene (ene)</th>
<th>Initiator</th>
<th>Thiol</th>
<th>Borohydride</th>
</tr>
</thead>
<tbody>
<tr>
<td>19</td>
<td>EGDMA</td>
<td>AIBN</td>
<td>Cys-Cl</td>
<td>No</td>
</tr>
<tr>
<td>20</td>
<td>EGDMA</td>
<td>AIBN</td>
<td>Cys-Cl</td>
<td>Yes</td>
</tr>
<tr>
<td>21</td>
<td>EGDMA</td>
<td>K$_2$S$_2$O$_8$</td>
<td>Cys-Cl</td>
<td>No</td>
</tr>
<tr>
<td>22</td>
<td>EGDMA</td>
<td>K$_2$S$_2$O$_8$</td>
<td>Cys-Cl</td>
<td>Yes</td>
</tr>
</tbody>
</table>

FTIR analyses of the final solid products of reactions performed with AIBN and potassium persulfate are shown respectively in Figure 17 and Figure 18. Results indicate that double bonds contained in EGDMA (1641 cm$^{-1}$) apparently were not completely consumed and that C-S bonds could not be detected (692 cm$^{-1}$). Incomplete consumption of vinyl bonds is possibly related to the fact that the main reagents were present in different phases. Based on the FTIR spectra, it is possible to infer that chloroalkanes were formed because of the characteristic band placed at 700 cm$^{-1}$ [63], indicating once more the formation of byproducts instead of the expected click reaction. It is also important to note that cysteine hydrochloride reduction with sodium borohydride did not lead to the expected high specificity of the click reactions, indicating that the results reported here were not related necessarily with the oxidation state of L-cysteine.

Thiol-ene Reactions with a Polymer Containing Pendant Double Bonds

In order to investigate the reasons why no effective click chemistry reaction was observed in the previous tests (with exception of the benchmark reaction presented in Section 4.1), a previously reported experiment was carried out. In the reported experiment, MPA was reacted with unsaturated polystyrene in presence of AIBN at 80 °C and in solution of dichloromethane [42]. Authors reported 87% conversion after 4 h of reactions [42]. For this reason, MPA and poly(styrene-co-ethylene glycol dimethacrylate) were selected as the thiol-ene pair and AIBN was used as thermal initiator at the same conditions reported previously. Besides
dichloromethane, two common additional solvents were also tested, as described in the experimental section.

The preparation of the copolymer material is described in Section 2.2.4. EGDMA was chosen as a comonomer because it is a crosslinking agent widely used for biomedical applications [64]. It was expected that at least a fraction of the double bonds of the final copolymer chain would be available after the copolymerization for later use in thiol-ene click reaction and bioconjugation. This hypothesis is consistent with the theory of FLORY [65] and STOCKMAYER [66], who proposed that chain crosslinking takes place in three steps: (i) linear structures are formed having pendant side groups; (ii) the polymer structure grows and branches begin to appear; (iii) crosslinking finally occurs. This strategy was already proven to be efficient by other authors who also used unsaturated copolymers in their researches [42,67]. There are many published works about styrene copolymerizations with EGDMA, but to the best of our knowledge there are no references to the presence of pendant double bonds in these materials.

![Figure 17. FTIR spectra of the final solid products of runs 19 and 20.](image1)

![Figure 18. FTIR spectra of the final solid products of runs 21 and 22.](image2)
One of the reasons why styrene was selected as the main monomer to carry out the tests is the fact that the styrene polymerization kinetics is well-known and can also be used for benchmark purposes [67,68]. For example, Figure 19 shows the monomer conversions in polystyrene (PS) and poly(styrene-co-ethylene glycol dimethacrylate) (P(S-co-EGDMA)) polymerizations. As one can observe in Figure 19, the reaction rates for the two reaction systems are very similar, mainly because only 0.5 wt% of EDGMA were added to the copolymerization system. Obtained results indicated that both reactions reached conversions of almost 100%. Therefore, in order to determine whether the intended pendant double bonds were present in the polymer structure, FTIR analyses were conducted and results are presented in Figure 20.

Figure 19. Comparison between monomer conversions for polystyrene and poly(styrene-co-ethylene glycol dimethacrylate).

Figure 20. FTIR spectra of PS and P(S-co-EGDMA).
As one can note in Figure 20, the FTIR spectra of the produced polymers were very similar and this is not difficult to explain, since only 0.5 wt% of EGDMA were added into the reacting mixture. However, an increase in the band located at 1723 cm\(^{-1}\) can be observed. IR bands of \(-\text{unsaturated and benzoate esters occur at } 1730-1715 \text{ cm}^{-1}\) [69]. Therefore, the band located in the copolymer at 1723 cm\(^{-1}\) probably refers to double bonds present in \(-\text{unsaturated esters}. This indicates that EGDMA was inserted in the polymer chains and that there are pendant double bonds in the copolymer structure. Accordingly, the copolymer produced is suitable for thiol-ene reactions that are presented in Table 4.

Table 4. Reactions performed with P(S-co-EGDMA) and MPA as the thiol-ene pair.

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Alkene (ene)</th>
<th>Initiator</th>
<th>Thiol</th>
<th>Solvent</th>
</tr>
</thead>
<tbody>
<tr>
<td>23</td>
<td>P(S-co-EGDMA)</td>
<td>AIBN</td>
<td>MPA</td>
<td>DCM</td>
</tr>
<tr>
<td>24</td>
<td>P(S-co-EGDMA)</td>
<td>AIBN</td>
<td>MPA</td>
<td>THF</td>
</tr>
<tr>
<td>25</td>
<td>P(S-co-EGDMA)</td>
<td>AIBN</td>
<td>MPA</td>
<td>DMF</td>
</tr>
</tbody>
</table>

Click reactions were performed in different toxic solvents, as commonly reported in the literature. FTIR analyses of the final products are shown in Figure 21. It is important to highlight that reactions 23 and 24 were performed in homogeneous media, while reaction 25 was performed in heterogeneous media, because the polymer was not soluble in DMF. Results presented in Figure 21 indicate that the band that refers to the pendant double bonds (1723 cm\(^{-1}\)) remains in the polymer structure after reactions 23 and 25, which is an indication that the thiol-ene bonds were not formed. For reaction 24, consumption of the double bonds may possibly have occurred; however, due to the interference of the polymer on the band that characterizes the C-S bonds (692 cm\(^{-1}\)), it was not possible to confirm unequivocally the occurrence of the click reaction.

It is important to highlight that reaction 23, for which the click reaction did not occur, was similar to a reaction reported as successfully performed by other research group [42]. The main difference was the composition of the polymer material that contained the pendant double bonds, poly(styrene-allyl bromide). However, this difference was not expected to interfere with the final results, as the P(S-co-EGDMA) used in the present work also contained ene groups and was also soluble in DCM. However, once more this indicates that click chemistry reactions depend on the nature and concentration of the reacting mixture, even when the reactants constitute a homogeneous liquid phase.

**Thiol-ene Reaction with DVB**

After this very long series of click reaction experiments, reaction A was reproduced, although replacing styrene by DVB in order to observe how the thiol-ene reaction would occur when the vinyl monomer was replaced by a divinyl monomer. In order to maintain similar concentrations of vinyl bonds, 0.27 g of DVB were added to the reacting mixture, instead of 0.42 g of styrene as described in the experimental section. This new experiment was named reaction 26 and FTIR results are shown in Figure 22.
Figure 21. FTIR spectra of reactions 23, 24, 25 and of P(S-co-EGDMA).

Figure 22. FTIR spectra of liquid and solid products of reaction 26 and of MPA, DMF and DVB for comparison.

As in reaction A, FTIR analyses were performed for the solid product and the residual liquid of reaction 26. Surprisingly, no indication of the characteristic C-S bond that characterizes the click thiol-ene reaction was observed at 692 cm$^{-1}$. Moreover, the residual liquid in this case was composed almost exclusively of DMF. Therefore, it seems clear that the source of the vinyl bond affects significantly the course of the click reaction.

GPC analysis of the solid product was performed and is presented in Figure 23. The obtained number average molar mass was equal to 302 g/gmol, with polydispersity index of 1.07.
Differently from what had been observed in reaction A, this time the average molar mass of the product did not correspond to the sum of the molar masses of DVB and MPA (130.19 g/gmol and 106.14 g/gmol respectively), being closer to the DVB dimer, especially if one considers the addition of a radical fragment produced by AIBN (NC₄H₆ = 68 g/mol).

![Normalized response (a.u.) vs Molar mass (g/gmol)](image)

**Figure 23.** Molar mass distribution of the product obtained in reaction 26.

Observing in more detail the UV result presented in Figure 24, one can see that there is a large band that was also detected with the RI detector and is associated to products with average molar mass of 302 g/gmol as already discussed. However, the UV detector also provided a small band, shifted towards lower molar masses. This band can probably be related to the presence of decomposed AIBN. AIBN typically presents an absorption band located in 365 nm [70]. But during AIBN decomposition, the traditional absorption band located in 365 nm and reported by the literature is gradually shifted towards lower wavelengths close to 255 nm [70,71], in the same region where the GPC analyses were conducted. Therefore, the small band presented in Figure 24 can probably be caused by the presence of decomposed AIBN, which would be in accordance with the extremely small molar mass observed.

However, the small band could also be related to the presence of residual reagents. To exclude this possibility, the reaction reagents were solubilized in THF and injected in the chromatograph. Results shown in Figure 25 and in Figure 26 indicate that none of the bands observed for Reaction 26 seem to be related to either DMF, MPA, AIBN or DVB, that present completely different profiles when compared to Reaction 26. Nevertheless, the fact that part of the AIBN seems to be decomposed and that its profile is different from the profile of Reaction 26 does not indicate that the small band observed in Figure 24 is not related to AIBN. As already discussed, the UV results reported in the literature clearly indicate that the absorption of AIBN changes during its decomposition reaction, and that the final result is a monomodal band detected ~255nm. So the small band present in Figure 24 is still probably associated to decomposed AIBN obtained after Reaction 26.
Finally, the UV signal provided by the GPC detector (shown in Figure 24) indicated the existence of multiple reaction products with molecular weight difference of approximately 150 g/mol, which clearly indicates that chain growth may take place and compete with the desired click reaction. Therefore, keeping a broad perspective, it is not possible to assure that the main click reaction constitutes the fastest and preferential reaction path in this complex reacting system in all cases. Perhaps the click reaction is not necessarily the preferred reaction path in most polymerization reaction systems.
Reaction of Styrene with L-cysteine

Styrene apparently exerts a fundamental role in the click reaction scheme among the analyzed reaction systems, since only the benchmark reaction A seemed to unequivocally produce thiolene products. Therefore, reaction 5 was reproduced, but replacing DVB by styrene in order to observe if the previously discussed issues (heterogeneous media, thiol reduction, initiator type, thiol:ene ratio) would still be important for other styrene reactions. FTIR spectra of the product of reaction 27 are shown in Figure 27. As marked in the graph, a band placed at 696 cm\(^{-1}\) could be detected for the product of reaction 27; however, due to the characteristic styrene and polystyrene bands at the same spectral region [72], unequivocal characterization of the C-S bands is not possible. Nevertheless, GPC analyses of the solid product could not be performed because the solid product was not soluble in the usual analytical solvents, which makes GPC and NMR analyses not possible.
Figure 27. FTIR spectra of the product of reaction 27 and styrene and L-cysteine for comparison purposes.

CONCLUSION

In the present work a series of experiments was conducted and multiple issues were raised concerning thiol-ene click reactions. According to the obtained results, it seems clear that the efficiency of thiol-ene click reactions depend on the particular reagents used to perform the reactions, including solvents, sources of vinyl bonds, sources of thiol groups and free-radical initiators. This particular issue has been largely overlooked in the open literature. In fact, the dienes used in the present manuscript (HD, DVB and EGDMA) apparently do not form thioether bonds in the tested conditions (heterogeneous reactions performed at mild temperature conditions), which are typical for conduction of bioconjugation. Even more important, in cases where occurrence of the thiol-ene click reaction was not observed, other side reactions (such as chain growth) took place and caused the formation of extremely complex mixtures of products. However, when the click reaction was successful (as in the benchmark reaction between styrene and MPA), the final product seemed to be very pure, which is in accordance with the click chemistry definition. Therefore, a successful choice of reagents and reaction conditions not only makes thiol-ene click chemistry possible, but also seems to inhibit other side reactions, as polymerization, for example.

Based on the obtained results, apparently thiol-ene click reactions should preferentially be conducted in homogeneous media, which is not clearly discussed in the literature and is prejudicial for most bioconjugation problems, where the unsaturated polymer matrix constitutes a solid phase and the protein is dissolved in the suspending medium. Also, it seems that the choice of the reactants and reaction conditions strongly impact the course of the click reaction, so that the reacting system must be carefully designed for the thiol-ene click reaction to be successful. As widely discussed throughout this text, click chemistry reactions are not always so simple as usually reported in the literature. It seems clear that many aspects of the click reaction chemistry must be discussed before these reactions can find widespread use for bioconjugation of biomolecules to polymer matrices.

Acknowledgments

The authors thank CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico, Brazil) and FAPERJ (Fundaçao Carlos Chagas Filho de Amparo à Pesquisa do Estado do Rio de Janeiro, Brazil) for supporting this work and providing scholarships.

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Print ISSN: 2055-0073(Print), Online ISSN: 2055-0081(Online)


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